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(71) Applicant *(for all designated States except US)*: INSTITUT PASTEUR [FR/FR]; 28, rue du Docteur Roux, F–75015 Paris (FR).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MOUNIER, Carine [FR/FR]; Bâtiment 7, 21, RPC Cloarec, F–92270 Bois–Colombes (FR). HACKENG, Tilman [US/US]; Scripps Research Institute, 10555 North Torrey Pines Road, SBR–5, La Jolla, CA 92039 (US). GRIFFIN, John [US/US]; Scripps Research Institute, 10555 North Torrey Pines Road, SBR–5, La Jolla, CA 92037 (US). BON, Cassian [FR/FR]; 7, boulevard Arago, F–75013 Paris (FR).

(74) Agents: MARTIN, Jean–Jacques et al.; Cabinet Regimbeau, 26, avenue Kléber, F–75116 Paris (FR).

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(57) Abstract

The present invention relates to $hsPLA_2$ gr II and to specific $hsPLA_2$ gr II peptides exhibiting an anticoagulant effect, to antibodies which are directed against said peptides and to pharmaceutical compositions comprising said peptides or antibodies. The present invention further relates to methods of regulating the coagulant effect and to methods of treating or preventing thrombus formation and limiting platelet activation *in vivo* in human or in animal comprising the step of administering an effective amount of said peptides. The present invention further relates to methods of screening new pharmaceutical compounds which may be used for the prevention or treatment of hemostatic disorders and to kits for the determination of hemostatic disorders.

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$HsPLA_2$ GR II PEPTIDES EXHIBITING AN ANTICOAGULANT EFFECT

The present invention relates to $hsPLA_2$ gr II and to specific $hsPLA_2$ gr II peptides exhibiting an anticoagulant effect, to antibodies which are directed against said peptides and to pharmaceutical compositions comprising said peptides or antibodies. The present invention further relates to methods of regulating the coagulant effect and to methods of treating or preventing thrombus formation and limiting platelet activation *in vivo* in human or in animal comprising the step of administering an effective amount of said peptides. The present invention further relates to methods of screening new pharmaceutical compounds which may be used for the prevention or treatment of hemostatic disorders and to kits for the determination of hemostatic disorders.

The human group II secretory phospholipase A_2 ($hsPLA_2$ grII) has been detected in various cellular types including macrophages, eosinophiles and blood platelets (Dennis, 1991). In platelets, hsPLA2 grII is associated with the α-granules and is released into the extracellular medium upon activation (Horigome et al., 1987; Kramer et al., 1989). The $hsPLA_2$ grII shares common characteristics with other group II secretory PLA_2 ($sPLA_2$). In particular, its polypeptide sequence is homologous to that of these enzymes and its mechanism of action is identical (Kramer et al., 1989 ; Seilhamer et al., 1989; Davidson and Dennis, 1991; Wery et al., 1991; Scott et al., 1991; Ami and Ward, 1996). The $hsPLA_2$ grII attracted particular attention in the cases of inflammatory diseases since its level into body fluids correlated with the severity of the pathological states (Mukherjee et al., 1992; Glaser et al., 1993; Pruzanski et al., 1993). It has been proposed that $hsPLA_2$ grII may be involved in the degradation of bacteria (Elsbach and Weiss, 1993), in exocytosis/degranulation processes (White et al., 1993; Murakami et al., 1993) and in the production of eicosanoids by stimulated inflammatory cells (Kurihara et al., 1991; Hara et al., 1991; Murakami et al., 1991; Suga et al., 1993).

The effect of hsPLA$_2$ grII on blood platelets functions has also been examined. It has been first demonstrated that this enzyme does not participate in the production of eicosanoids during platelet activation (Mounier et al., 1993) and does not interfere with platelet stimulation once secreted (Mounier et al., 1994). Blood platelet activation plays a central role during hemostasis leading to primary plug formation and increasing the efficiency of coagulation process (Mann et al., 1990; Davie et al., 1991; Zwall et al., 1992; Davie, E.W., 1995). Blood platelets are also a source of factor V (Tracy et al., 1982). It is thus tempting to suggest a role for hsPLA$_2$ grII on blood coagulation, once secreted by activated platelets, The prothrombinase complex composed of FVa, FXa, phospholipids, and Ca++ plays a central role in the coagulation cascade (Mann et al., 1990; Rosing et al., 1988). The hsPLA$_2$ grII is shown to exert a moderate anticoagulant effect on plasma (Cirino et al., 1993) and to inhibit prothrombinase activity (Inada et al., 1994). Using purified recombinant hsPLA$_2$ grII, these observations have been recently confirmed and its inhibitory mechanism has been further examined. It has been demonstrated that the anticoagulant effect of hsPLA$_2$ grII was phospholipid-independent, leading to the hypothesis that its molecular target might be a protein component, most likely FXa or FV/Va (Mounier et al., 1996).

Further studies of sPLA2 revealed that although all sPLA2 have a common active site, different sPLA2 were shown to have distinct pharmacological sites explaining the diversity of pharmacological effects of venom sPLA2 such as neurotoxicity, myotoxicity, cardiotoxicity, platelet aggregation inhibition or potentiation and anticoagulant action (Ouyang et al., 1992; Kini and Evans, 1989). In particular, all venom anticoagulant sPLA$_2$ have a basic pI correlated with the presence of basic amino acids located between residues 50-80 (Kini and Evans, 1987; Verheij et al., 1980).

As mentioned above, these studies did not indicate which component of coagulation may be affected by hsPLA$_2$

grII and the region of the hsPLA$_2$ grII which is independently able to inhibit prothrombinase activity.

Moreover, these studies did not indicate under which suboptimal conditions the hsPLA$_2$ grII inhibitory effects are obtained on prothrombinase activity.

Thus, it would be desirable to identify the specific part of the hsPLA$_2$ grII which is involves as an inhibitor of prothrombinase activity and to identify the prothrombinase complex component which is affected in this inhibition mechanism. Indeed, a need exists for new peptides having anticoagulant activity which can be used for therapeutic purposes as anticoagulants.

The present invention is based upon the discovery of the precise hsPLA$_2$ grII region which is specifically involved in the inhibitory effects on prothrombinase activity and its anticoagulant mechanism. Further, the Applicant has clearly identified the proteinaceous target of hsPLA$_2$ grII, the prothrombinase complex component which is affected, and under which conditions these effects can be achieved.

Thus the invention relates to a peptide comprising at least eleven amino acids numbered 51 to 62 of hsPLA$_2$ gr II sequence shown in table 2.

In another embodiment, the invention relates to a peptide according to the invention, comprising amino acids numbered 51 to 74 of hsPLA$_2$ gr II sequence.

The present invention also relates to a peptide exhibiting an anticoagulant effect corresponding to an amino acid chain containing at least a seven consecutive amino acid from the numbered 51 to 62 amino acid sequence of hsPLA$_2$ gr II.

The invention further comprises a peptide exhibiting an anticoagulant effect corresponding to an amino acid chain containing at least 14 amino acids having at least 50% amino acids identity with the numbered 51 to 74 amino acid sequence of hsPLA$_2$ gr II.

Peptides according to the invention include those peptides mentioned above and peptides with minor amino acid

variations from the natural amino acid sequence of the peptide; in particular, conservative amino acid replacements are contemplated.

Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids are generally divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) non-polar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cystine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. For example, it is reasonably predictable that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar conservative replacement of an amino acid with a structurally related amino acid will not have a major effect on the biological activity. Peptide molecules having substantially the same amino acid sequence as the peptides peptide according to the invention but possessing minor amino acid substitutions that do not substantially affect the functional aspects are comprise in the present the invention.

In a particular embodiment, the invention relates to a peptide according to the invention, wherein said peptide is able to inhibit prothrombinase activity.

In a preferred embodiment, the invention also relates to a peptide according to the invention, wherein the molecular target for the anticoagulant action of said peptide is Factor Xa (Fxa).

The invention further relates to a peptide according to the invention, wherein the presence of Factor Va (Fva) is capable of reversing the activity of said peptide, particularly under suboptimal conditions.

The invention further relates to peptide according to the invention, wherein said peptide is a Fva competitor.

The peptides according to the invention can be prepared by peptide synthesis or by recombinant DNA techniques, which are known to the person skilled in the art.

The nucleotide sequences (RNA or DNA) coding for the peptide according to the invention are part of the invention.

The invention further relates to anticoagulant compound, preferably prothrombinase activity inhibitor compound, more preferably fXa inhibitor compound, and Fva competitor compound selected in a group comprising the peptides according to the invention.

The term « Fva competitor compound » refers to compound according to the invention which either may bind to FXa at the same site(s) as FVa, or that the binding of FVa to FXa may modify the structure of FXa leading to the dissociation and the removal of said Fva competitor compound.

In another aspect of the invention, the invention comprises a monoclonal or polyclonal antibody, or fragments thereof, characterized in that it binds a peptide according to the invention.

Accordind to a particular embodiment of the present invention, said monoclonal or polyclonal antibody, or fragments thereof according to claim 9 are characterized in that it inhibits hsPLA$_2$ gr II anticoagulant effect.

The peptides according to the invention may also be used as antigenic models for the preparation of antibodies or antibodies like proteins, which may be used to inhibit excess activity of endogenous substances.

The monoclonal or polyclonal antibody according to this invention includes any naturally or non-naturally occurring polypeptide having the binding specificity of peptides according to the invention, that is, a polypeptide which binds to an epitope on said peptides, inhibits said peptides binding with Fxa or antagonizes the said peptides anticogulant effect. Examples of such antibody include a half antibody molecule (a single heavy: light chain pair), or a fragment, such as the univalent fragments Fab or Fab'

and the divalent fragment F(ab')2 ("FAB" meaning fragment antigen binding), that possess the same specificity for binding as complete antibody. A fragment, according to the present invention may also be a single chain Fv fragment produced by methods well known in the art. See Skerra et al., Science, 240: 1038-1041 (1988) and King et al., Biochemical J., 290: 723-729 (1991), each of which is hereby incorporated by reference. The antibody of the present invention also includes anti-idiotypic antibodies produced by methods well-known to the art of the invention. See, e.g. Cozenza, Eur. J. Immunol. 6: 114 (1976) and Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor publications pp. 726 (1988), each of which is hereby incorporated by reference.

The term "epitope" as used in describing this invention, includes any determinant of peptides according to the invention responsible for the specific interaction with an antibody molecule. Epitopic determinants usually consist of chemically active surface groupings of amino acids and have specific three-dimensional structural characteristics, as well as specific charge characteristics.

The antibody according to this invention also includes antibody conjugates, which are for example, enzymes, fluorescent markers, radiolabels.

In another embodiment, the monoclonal antibody of the present invention is a "humanized" antibody, produced by techniques well-known in the art. Carter et al., PNAS 89: 4285-4289 (1992); Singer et al., J. Immun. 150: 2844-2857 (1992) and Mountain et al. Biotechnol. Genet. Eng. Rev. 10: 1-142 (1992), each of which is hereby incorporated by reference.

Monoclonal antibodies can be produced in various ways using techniques well-understood by those having ordinary skill in the art. Details of these techniques are described in Antibodies : A Laboratory Manual, Harlow et al., Cold Spring Harbor Publications, p. 726 (1988), which is hereby incorporated by reference.

The subject of the present invention is also a pharmaceutical composition comprising a peptide or an antibody according to the invention in combination with a pharmaceutically acceptable vehicle.

In another further aspect of the present invention, the invention relates to the use of a peptide or an antibody according to the invention in a manufacture of a medicament for the prevention or the treatment of hemostatic disorders.

The human type PLA_2 grII and derivatives of the present invention can be used for therapeutic purposes as anticoagulants. The peptides can be used alone, or they can be used in combination with other drugs.

Another subject of the present invention is a method of regulating the coagulant effect *in vivo* in human or in animal comprising the step of administering an effective amount of an active peptide, of an antibody or of a pharmaceutical composition according to the invention.

According to another aspect, the invention relates to a method of treating or preventing thrombus formation and limiting platelet activation *in vivo* in human or in animal comprising the step of administering an effective amount of an active peptide or of a pharmaceutical composition according to the invention.

The pharmaceutical composition of the present invention may be administered in the form of oral, intravenous, intraperitoneal, or intra muscular administration, transdermal diffusion, and others. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For example, pharmaceutically acceptable carriers include, water, saline, buffers, solutions, non-toxic excipients, including salts, preservatives, buffers and the like, as described, for instance, in Reminington's Pharmaceutical Sciences, 15th Ed. Easton: Mack Publishing Co. pp 1405-1412 and 1461-1487 (1975) and The National Formulary XIV., 14th Ed. Washington: American Pharmaceutical Association (1975), the contents of which are hereby incorporated by reference. Examples of non-aqueous solvents are propylene glycol,

polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, and other compounds described, e.g., in Merck Index, Merck & Co., Rahway, New Jersey. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to routine skills in the art. See Goodman and Gilman's, The Pharmacological Basis for Therapeutics (7th Ed.).

The quantity of the peptide or antibody of the present invention necessary for effective therapy will depend upon many different factors, including the means of administration, target site, physiological state of the patient, other medicants administered, etc. Thus treatment dosages should be titrated to optimize safety and efficacy. Typically, dosages used in vitro may provide useful guidance in the amounts useful for in situ administration of the peptide or the antibody, and as noted above, animal models may be used to determine effective dosages for treatment of particular disorders. Various considerations are described, e.g. in Gilman et al. (eds.) (1990) Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th Ed., Pergamon Press; and Remington's Pharmaceutical Sciences, 17th Ed. (1990), Mack Publishing Co., Easton, Pa., each of which is herein incorporated by reference.

The peptides of the present invention may also be used to characterize new drugs, either as molecular models or as tools for screening.

Thus, in another further aspect, the present invention relates to method of screening new compounds for their use as medicament for the prevention or the treatment of coagulation disorders, comprising the use of a peptide according to the invention.

In a particular embodiment, a method of screening according to the invention comprises the steps of :

a) contacting a sample containing said test compound with a peptide according tothe invention;

b) detecting the binding of said test compound with said peptide ; and

c) selecting said test compound which is able to bind with said peptide.

In another particular embodiment, a method of screening according to the invention comprises the steps of :

a) contacting a sample containing said test compound with a peptide according to the invention in conditions permitting the measure of said peptide anticoagulant effect;

b) measuring the said peptide anticoagulant effect ; and

c) selecting said test compound which is able to modify said peptide anticoagulant effect, particularly test compound which is able to inhibit said peptide anticoagulant effect.

The peptides of the present invention may also be use to develop kits for detecting hemostatic disorders.

Thus, the present invention also includes kits for the determination of a hemostatic disorder in a sample from human or animal, comprising a peptide according to the invention.

Other advantages and features of the present invention will become apparent in the light of the examples which follow.

Brief description and legend of the Figures

Figure 1: Effect of $hsPLA_2$ grII on FXa and on FVa activities in clotting assays.

(A) FXa-1-stage clotting assay. 10 nM FXa in Hepes buffer is incubated at 37°C in the absence (-0-, control) or in the presence of 3.5 μM hsPLA2 grII (-●-). After indicated times, 20 μl of the incubation mixture is used to perform

an FXa-1-stage assay as described in "Methods". The amount of active FXa is determined using a calibration curve of purified FXa. The mean of duplicate experiments are shown. (B) Prothrombin time clotting assays are performed as described in "Material and Methods" in the absence (-0-) or in the presence of 3.5 μM $hsPLA_2$ grII (-●-). The FVa activity is determined using a calibration curve of purified FVa. The means ± SEM of three independent experiments are shown.

Figure 2 : Effect of $hsPLA_2$ on FXa and on FVa activities in coagulation assays. For FXa-one-stage coagulation assay, 20 nM FXa in Hepes buffer is incubated for 2 min at 37°C, in the absence (-0-) or in the presence (-●-) of 5 mM CaCl2, with the indicated amounts of $hsPLA_2$. Then, 20 μl of the incubation mixture is used to perform an FXa-one-stage assay as described in " Methods ". The amount of active FXa is determined using a calibration curve of purified FXa. The activity of FVa is measured using prothrombin time coagulation assays, performed as described in "Methods", in the presence of 5 mM CaCl2 and the indicated amounts of hsPLA2 (-▲-). The FVa activity is determined using a calibration curve of purified FVa. The means ± SEM of three independent experiments are shown.

Figures 3 and 3 bis: Effect of hsPLA2 grII on prothrombinase activity.
Prothrombinase assays are performed as described in "Methods", after $hsPLA_2$ grII is preincubated with 120 pMFV and 20 pM FXa (A), with 20 pM FXa(B), or with 120 pM FV (C). Production of thrombin is followed in the absence (-●-) or in the presence of 35 nM $hsPLA_2$ grII (-□-), 350 nM $hsPLA_2$ grII (-Δ-) or 3.5 μM $hsPLA_2$ grII (-0-). The means from duplicate experiments are shown.

Figure 4: Effect of $hsPLA_2$ grII on prothrombinase activity measured under suboptimal conditions.

Prothrombinase assays are performed as described in "Methods" in the absence (-●-) or in the presence of 3.5 μM $hsPLA_2$ grII (-0-).
(A) Prothrombinase activity (20 nM FXa, 120 pM FV, 5 mM Ca++) measured in the absence of PL; (B) prothrombinase activity (2 nM FXa, 5 μM phospholipid (PL), 5 mM Ca++) measured in the absence of FV or FVa. The means ± SEM of three independent experiments are shown.

Figure 5 : Effect of $hsPLA_2$ on prothrombinase activity in the absence of PL. Prothrombinase assays are performed as described in "Methods" in the presence of the indicated amount of $hsPLA_2$.
(A) Prothrombinase activity (20 nM FXa, 120 pM FVa, 5 mM Ca++) is measured in the absence of PL (-0-) and 20 nM FXa with 5 mM Ca++ served as control (-●-).
(B) Prothrombinase activity (1 nM FXa, 1 nM FVa, 5 mM Ca++) is measured in the absence of PL (-0-) and 1 nM FXa with 5 mM Ca++ served as control (-●-). The means ± SEM of three independent experiments are shown.

Figures 6 and 6 bis: Reversal of $hsPLA_2$ grII inhibition on prothrombinase activity by the addition of FVa during the prothrombinase assay.
Prothrombinase activity assays (20 pM FXa, 120 pM FV, 5 μM PL, 5 mM Ca++) are performed as described in "Methods", either in the absence of hsPLA2 grII (-●-), or, in the presence of 3.5 μM $hsPLA_2$ grII with 200 pM (-0-), 20 pM (-Δ-), 4 pM (-□-), or 0 pM (-▲-) FVa (FVa is added at 6 min). The means from duplicate experiments are shown.

Figure 7: Inhibition of prothrombinase activity by peptide 51-74.
Prothrombinase activity assays are performed as described in "Methods" with 20 pM FXa, 120 pM FV, 5 μM PL and 5 mM Ca++, in the absence (-●-) or in the presence of 4 μM (-□-), 13 μM (-0-) or 26 μM (-Δ-) of peptide 51-74. The means from duplicate of a typical experiment are shown.

Figure 8 : Calorimetric titration of FXa with hsPLA2 at 37°C. The top panel shows the heat signal (after subtraction of base line) for 21 injections of 8 μL aliquots of buffer (20 mM Tris-HCl, pH 7.4, 0.125 M NaCl and 5 mM CaCl2) with 45.7 μM $hsPLA_2$ into a 1.35 mL cell containing the same buffer with 4.1 μM FXa. The bottom panel shows the integrated heat of each injection after correction for the heat of dilution of $hsPLA_2$ and normalization to the amount of $hsPLA_2$ injected (filled rectangles). The curve through the points represents the best fit to a model involving a single set of independent binding sites. The apparent thermodynamic parameters describing the fit are N = 0.95, Kd = 230 nM, and $\Delta H°$ = - 4.6 kcal/mole.

EXAMPLES

Materials

Human prothrombin (FII) is purified according to DiScipio and Davie (1979). Human factor V (FV) is purified as described (Kane and Majerus, 1981) with minor modifications (Hackeng et al., 1994). Activation of human FV is performed as described previously (Hackeng et al., 1994). FIXa (activated factor IX), FX (factor X) and Fxa (activated factor X), are from Enzyme Research Laboratories, South Bend, IN. Recombinant FVIII is a kind gift of Dr. Roger Lundblad of Baxter Hyland, Duarte, CA. The chromogenic substrate for thrombin amidolytic activity, CBS 34,47, is from Diagnostica Stago (Asnières, France) and Chromogenix (Cincinnati, OH). FV-deficient plasma is from George King Bio-Medical (Overland Park, KS). Innovin is from DADE (Miami, FL), BSA (bovine serum albumin) (fraction V) is from Sigma (St Louis, MO). The synthetic scrambled peptide containing $hsPLA_2$ residues 51-74 as well as peptides 51-62, 59-70, 63-74, 62-51 (reverse) and D-51-62 (all residues in the D-configuration) are from Neosystem, Isochem SA (Strasbourg, France). The synthetic peptide 51-74 of the $hsPLA_2$ grII (human group II secretory phospholipase A_2) is purchased to the organic chemistry unit of Pasteur Institute (Paris, France). The peptides

are purified by HPLC (purity ≥ 95%), their purity and sequence checked by mass spectroscopy. All these peptides have a N-terminal acetyl group and a C-terminal amide group.

Methods

Preparation of recombinant $hsPLA_2$ grII

The expression plasmid used is pT7-7 (Pharmacia) which is then transfected into the BL21[DE3]Coli strain [Studies & Moffat, 1986]. The BamHl/Hindlll fragment encoding $hsPLA_2$ grII [Franken et al., 1992] is cloned in the similar cut expression vector. The $hsPLA_2$ grII is expressed as a fusion protein with a 6 amino acids N-terminal extension ending in an arginine residue for tryptic liberation of the PLA_2 (phospholipase A_2) moiety. Cells are grown in LB medium enriched with M9 salts. An overnight culture is diluted in fresh medium (1 to 10) and after 2 hours induced with 0.4 mM IPTG. Cells are harvested 4 hours after induction. The inclusion bodies are then obtained by centrifugation and the protein is subsequently sulphonated. After dialysis and lyophilization, the sulphonated protein is reoxidized for 48 hours at 4°C in 0.9 M guanidine HCl, 10 mM $CaCl_2$, 8 mM cysteine, 1 mM cystine, 10 mM borate buffer pH 8.5. Subsequently, the active $hsPLA_2$ grII is obtained tryptic cleavage of the fusion protein.

The $hsPLA_2$ grII is purified on two subsequent SP-sephadex columns at pH 6 and pH 7.5. The purified $hsPLA_2$ grII is then tested for its activity, using a fluorescent substrate, and checked for its purity by FPLC chromatography and SDS-PAGE as already reported [Mounier et al., 1994].

Prothrombinase assay

Phospholipid (PL) vesicles are prepared essentially as described by de Kruijff et al. [1974]. Solutions of phosphatidylserine and phosphatidylcholine (ratio of 1:9, PS:PC) in chloroform are mixed vigorously and dry under nitrogen. The dried PL are resuspended as vesicles in 0.1

M Tris-HCl, 0.05 M NaCl, pH 7.4 by sonication for 10 minutes.

Prothrombinase complexes are reconstituted using purified components, at 37°C and in Tris-buffered saline (0.1 M Tris-HCl, 0.05 M NaCl, 0,5% BSA, 5 mM $CaCl_2$, pH 7.4) under the following conditions:

a) FV, FXa and PL:

- In FXa/FV-preincubation conditions, 20 pM FXa is incubated with 120 pM FV for 4 min, then the reaction is started with 5 μM PL and 200 nM FII.
- In FV-preincubation conditions, 10 pM FV is incubated for 4 min, then the reaction is started with 1 nM FXa, 5 μM PL and 200 nM FII.
- In FXa-preincubation conditions, 10 pM FXa is incubated for 4 min, then the reaction is started with 1 nM FV, 5 μM PL and 200 nM FII.

b) FV, FVa and FXa: 20 nM FXa is incubated for 4 min with 120 pM FV (or 120 pM FVa), then the reaction is started with 1 μM FII (prothrombin).

c) FXa and PL: 2 nM FXa is incubated for 4 min, then the reaction is started with 5 μM PL and 1 μM FII.

d) FVa, FXa, and PL; the same experimental procedure is followed as for FXa/FV-preincubation conditions except that 120 pM FV is replaced by 20 pM FVa.

After the addition of prothrombin, aliquots are taken at various timepoints and the reaction is immediately stopped by the addition of 10 mM or 50mM EDTA (final concentration). The level of activated prothrombin activity is determined by hydrolysis of the chromogenic substrate CBS (100 μM or 300 μM, final concentration), monitored at 405 nm, and expressed in terms of thrombin concentration, using a calibration curve established with purified α-thrombin.

To determine the effect of $hsPLA_2$ grII or synthetic peptides on prothrombinase activity under these different conditions, indicated amounts of these compounds (or control buffer) are added to the reaction mixture before the 4-min preincubation period.

Addition of FVa during prothrombinase assays

FXa/FV-preincubation conditions are used as described above (a). Briefly, 20 pM FXa is incubated with 120 pM FV for 4 min, then the reaction is started with 5 μM PL and 200 nM FII. After 6 min, the indicated amounts of FVa are added. The formation of thrombin as a function of time is followed as described above.

Intrinsic tenase assay

Intrinsic tenase purified protein components are mixed at 37°C using purified components in Tris-buffered saline under the following conditions. 25 nM FIXa is incubated for 5 min with 1 nM FVIII in the absence or in the presence of defined $hsPLA_2$ amounts, then FX activation is started by addition of 1 μM FX (final concentrations). Aliquots are then taken at various time points and the reaction is immediately stopped by the addition of aliquots containing 50 mM EDTA (final concentration). The level of FXa activity is determined by hydrolysis of the chromogenic substrate S-2222 (200 μM, final concentration) monitored at 405 nm, in comparison to a standard curve using purified FXa.

FXa-one-stage clotting assay

FXa (20 nM) is incubated at 37°C in Hepes-buffered saline (50 mM Hepes pH 7.4, 0.1% BSA, 0.1 M NaCl) in the absence or in the presence of 5 mM $CaCl_2$ for different limes and in the absence (control) or in the presence of various concentrations of $hsPLA_2$ grII. After incubation times, FXa-one-stage coagulation assays are performed as follows: 20 μl of the incubation mixture is added to a prewarmed mixture of 25 μl FV-deficient plasma and 30 μl PS:PC at 166 μM. After 1 minute, coagulation is started by the addition of 50 μl CaCl2 at 20 mM. Clotting time is recorded using an ST4 coagulometer (Diagnostica stago, Asnières, France).

FVa activity measured in a prothrombin time clotting assay

Fva (1 nM) is incubated for 2 min at 37°C in Hepes-buffered saline containing 5 mM $CaCl_2$ in the absence (control) or in the presence of various concentrations of $hsPLA_2$ grII or 110 μM basic peptide (residues 51-74 of the $hsPLA_2$ grII). After various incubation times, a

prothrombin time assay is performed as follows: 5 µl of the incubation mixture is added to a prewarmed mixture of 50 µl FV-deficient plasma and 45 µl Hepes-buffered saline. After 1 minute, coagulation is started by the addition of 50 µl innovin. Clotting times are recorded using an ST4 coagulometer.

Measurement of FV activation by thrombin or FXa

A) FV (300 nM) is incubated for 30 minutes in Hepes-buffered saline (50 mm Hepes pH 7.4, 0.1% BSA, 0.1 M NaCl, 5 mM $CaCl_2$) at 37°C with or without 3.5 µM $hsPLA_2$ grII, then the activation is started by the addition of 1 nM thrombin. During 60 minutes, the amounts of generated FVa are determined by performing a prothrombin time clotting assay as described above, and FV proteolysis is also analysed by SDS-PAGE.

B) FV (3 nM) is incubated for 30 minutes in Hepes-buffered saline (50 mm Hepes pH 7.4, 0.1% BSA, 0.1 M NaCl, 5 mM CaCl2) at 37°C in the presence of 25 µM PL, and with or without 3.5 µM $hsPLA_2$ grII, then the activation is started by the addition of 6 nM FXa. During 120 minutes, the amounts of generated FVa are determined by performing a prothrombin time clotting assay as described above.

Isothermal titration calorimetry (ITC)

Experiments are carried out on the MicroCal MCS ultrasensitive titration calorimeter (MicroCal Inc., Northampton, MA) using the OBSERVER software provided by the manufacturer for instrument control and data acquisition (Wiseman et al., 1989). To improve base line stability, the temperature of the system is kept at 5°C below the temperature of the actual experiment with a water bath, and temperature is equilibrated for 12 h. During a titration experiment, the FXa sample is thermostated at 37.0 ± 0.1°C in a stirred (410 rpm) reaction cell (1.3514 ml), and 31 injections, each of 8 µl volume and 5 s duration, with a 3.5 min interval between injections, are carried out using a 250-µl syringe filled with a $hsPLA_2$ solution. An injection series is preceeded by a 2 µl calibration injection. The reference cell of the calorimeter contained water plus 0.01% sodium azide. Data

points are averaged and stored at 2-s intervals. All buffer solutions (Tris-HCl 20 mM, pH 7.4, NaCl 0.125 M and $CaCl_2$ 5 mM) are thoroughly degassed by stirring under vacuum before use. Protein samples are prepared in buffer of the same batch to minimize artifacts due to any differences in buffer composition. Titration experiments are performed with 1.5, 3.5 and 4.1 μM FXa and corresponding concentrations of hsPLA2 in the syringe, ensuring a final $hsPLA_2$/FXa mole ratio of 2:1 in the reaction cell. Raw calorimetric data, i.e. heats absorbed or released accompanying the addition of aliquots of the hsPLA2 solution into the FXa solution, are processed using the software package ORIGIN (Wiseman et al., 1989, Lin et al., 1994). The area under the resulting peak following each injection is proportional to the heat of interaction Q. When corrected for the titrant dilution heat and normalized to the concentration of added titrant, Q is equal to the binding enthalpy $\Delta H'_b$ at that particular degree of binding. The calorimetric binding isotherm is fitted by an iterative nonlinear least squares algorithm (Marquardt method) to a binding model employing a single set of independent sites. The association (K_a) and dissociation (K_d) constants, molar binding stoichiometry (N), and molar binding enthalpy ($\Delta H°$) are determined directly from the fitted curve. The Gibbs free energy and molar entropy of binding are calculated using the equations $\Delta G° = -RT \text{ Ln } K_a$ and $\Delta S° = (\Delta H° - \Delta G°)/T$, respectively, where R is the gas constant and T is the absolute temperature in degrees Kelvin.

Surface plasmon resonance (SPR) experiments

Studies are performed using a BIACORE® 2000 system (Biacore AB, Uppsala, Sweden). Reagents, including surfactant P20, the amine coupling kit containing N-hydroxysuccinimide, N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide, ethanolamine hydrochloride and Sensor Chips CM5 are supplied by Biacore. The immobilization of FXa on the sensor chip surface is performed as follows. 30 μl of FXa (14 μg/ml in 10 mM sodium acetate, pH 4.8) is covalently coupled via primary amino groups on a CM5 sensor

chip surface according to the manufacturer's description (Biacore). The immobilization run is performed at a flow of 5 µl/min at 25°C. The SPR signal for immobilized FXa (three different flow cells with three different quantities of FXa) are found to be: 4,400 resonance units (RU), 2,000 RU and 1,000 RU, where 1 RU corresponds to an immobilized protein concentration of ~ 1 pg/mm2. Unreacted moieties on the surface are blocked by ethanolamine. One independent flow cell of the same sensor chip, used as a control flow cell, is subjected to a "blank immobilization", i.e. with no FXa added. All experiments are carried out in 10 mM HEPES, pH 7.4, 0.005 % surfactant P20 and 150 mM NaCl. $hsPLA_2$, varying from 0-25 µg/ml, is injected in the same buffer in the presence or in the absence of CaCl2 at 5 mM, with a flow of 10 µl/min. Between each injection, surfaces are regenerated with 10 µl of 1 M NaCl. Analyses are performed at 25°C. Kinetic constants, kon (association rate constant) and koff (dissociation rate constant), for the interaction of $hsPLA_2$ with immobilized FXa are calculated using Biacore Biaevaluation 2.1 software using curve fitting to a simple two-component model of interaction (A + B = AB) for a titration of the solution $hsPLA_2$-immobilized FXa (Nieba et al., 1996). Values, for a series of FXa/$hsPLA_2$ complexes, are determined after subtraction of control signals obtained from the injection of various $hsPLA_2$ concentrations on the control flow cell.

Statistical analysis

The significance of the data is evaluated with the Student's t-test for unpaired data, NS, non-significant; * $p<0.05$; ** $p<0.005$, *** $p<0.001$.

EXAMPLE 1

Effect of $hsPLA_2$ grII on FXa and FVa activities measured in clotting assays (FIGURE 1)

We previously described a significant inhibitory action of $hsPLA_2$ grII both on plasma recalcification time experiments and on prothrombinase complex reconstituted with purified components. This inhibition was observed independent of the enzymatic activity of the enzyme,

leading to the hypothesis that hsPLA2 grII might affect FXa and/or FVa (Mounier et al., 1996).

The effect of $hsPLA_2$ grII on FXa activity is first examined. FXa-one--stage clotting assays, performed with FV-deficient plasma and fixed amount of FXa preincubated for different times with or without $hsPLA_2$ grII, offer good conditions to study the effect of this enzyme on FXa activity under more physiological conditions. Figure 1A shows a time-dependent inhibition of FXa activity by 3.5 μM $hsPLA_2$ grII, the inhibition being even faster when 5 mM Ca++ is present in the preincubation mixture (see Fig.2).

The effect of $hsPLA_2$ grII is next investigated on FVa activity measured with a prothrombin time clotting assay, using FV-deficient plasma. Fixed amounts of FVa are preincubated for different times with or without $hsPLA_2$ grII before addition to the assay. In these conditions, clotting efficiency is related to the activity of added FVa. Figure 1B shows that 3.5 μM $hsPLA_2$ grII is unable to significantly reduce the activity of FVa.

<u>Effect of $hsPLA_2$ grII on FXa and FVa activities measured in coagulation assays (FIGURE 2)</u>

FXa-one-stage coagulation assays, performed with FV deficient plasma and fixed amounts of FXa that had been preincubated with varying amounts of $hsPLA_2$, showed a dose-dependent inhibition of FXa activity by $hsPLA_2$ (Fig. 2). The inhibition is strongly reduced in the absence of Ca++ in the preincubation mixture, even though Ca++ is later present during FXa-one-stage assays (Fig. 2). This implies that the inhibitory effect of $hsPLA_2$ does not involve a Ca++-dependent action on PL, but rather an interaction with FXa that requires the presence of Ca++. The effect of $hsPLA_2$ on FVa activity is investigated with a prothrombin time coagulation assay using FV deficient plasma. Fixed amounts of FVa are preincubated with hsPLA2 before the assay, in which coagulation efficiency is related to the activity of FVa. Figure 2 shows that preincubation of $hsPLA_2$ with FVa does not reduce the activity of FVa.

Thus, these results show that $hsPLA_2$ does not inhibit FVa activity, whereas FXa is inhibited by $hsPLA_2$.

EXAMPLE 2

Inhibition of prothrombinase activity by $hsPLA_2$ grII

In order to study the effect of $hsPLA_2$ grII on prothrombinase activity the prothrombinase complex composition is varied to create optimal (FXa, FVa, PL and Ca++) and different suboptimal conditions(i.e. using FV instead of FVa, as well as in the absence of FVa or PL). When the prothrombinase complex is reconstituted from 20 pM FXa, 120 pM FV, 5 μM PL (phospholipid) and 5 mM Ca++, after that $hsPLA_2$ grII is preincubated with FXa and FV, a strong inhibition of prothrombinase activity is observed by low concentrations of $hsPLA_2$ grII (Fig. 3A and 3A bis). When PL concentrations are decreased to 1 μM, or increased to 50 μM, we did not observe any modification of the inhibitory activity of the $hsPLA_2$ grII (data not shown). These results are in good agreement with our previous report showing an IC50 (concentration of inhibitor producing 50% inhibition)of 30 nM under those experimental conditions and the PL independent action of the $hsPLA_2$ grII (Mounier et al., 1996). When $hsPLA_2$ grII is preincubated with either FXa (Fig. 3B and 3B bis) or FV (Fig. 3C and 3C bis), the inhibitory action of $hsPLA_2$ grII is much stronger for preincubation with FXa than with FV, supporting the observations made in coagulations assays.

Next, other suboptimal conditions of the prothrombinase complex are tested: 1) in the absence of PL, 2) in the absence of FV or Fva or 3) in the absence of both PL and FV or Fva.

Figures 4A and 4B represent the results obtained in the absence of PL (20 nM FXa, 120 pM FV, 5mM Ca++) and in the absence of FV or FVa (2 nM FXa, 5 μM PL, 5mM Ca++). For both conditions, $hsPLA_2$ grII induces an 50%-inhibition of the prothrombinase activity at 3.5 μM. This indicates that neither PL nor FV are required for the inhibitory effect of $hsPLA_2$ grII on prothrombinase activity,

supporting a hypothesis that the anticoagulant effect of $hsPLA_2$ grII might involve an interaction with FXa.

Figures 5A and 5B represent the results obtained in the absence of PL (20 nM FXa, 120 pM FVa, 5mM Ca++ ; or 1 nM FXa, 1 nM FVa, 5mM Ca++) and in the absence of both PL and FVa (20 nM FXa, 5mM Ca++ ; or 1 nM FXa, 5mM Ca++). The effect of $hsPLA_2$ on prothrombinase activity is then tested in the absence of PL. A potent inhibitory effect of hsPLA2 in the presence of FVa (Fig. 5A and 5B) can be seen. In contrast, the action of FXa on prothrombin in the absence of both PL and FVa is unaffected by $hsPLA_2$ (Fig. 5A and 5B), suggesting that $hsPLA_2$ does not interfere with direct prothrombin activation by FXa. Moreover, the amidolytic activity of FXa on its chromogenic substrate, S-2222, is not inhibited by $hsPLA_2$ (data not shown). The inhibition of prothrombinase (20 nM FXa, 120 pM FVa, 5 mM Ca++) by $hsPLA_2$ is never complete in the absence of PL, even at high concentrations of $hsPLA_2$ (10 μM), and as can be seen, the rate of prothrombin activation decreased until a value corresponding the same as that obtained with FXa alone, i.e. 0.16 ± 0.008 nM thrombin.min-1 compared to 0.14 ± 0.004 nM thrombin.min-1 (Fig. 5A). Under these suboptimal conditions, only a fraction of FXa is bound to FVa and the prothrombinase activity of free FXa is not inhibited by $hsPLA_2$. When assays are performed in the absence of PL under conditions that increase the ratio of FXa bound to FVa (1 nM FXa, 1 nM FVa, 5 mM Ca++), the percent of inhibition by $hsPLA_2$ is much higher, and at high hsPLA2 levels the prothrombin activation rate approached that seen for FXa alone (Fig. 5B).

The effect of $hsPLA_2$ on intrinsic tenase activity in the absence of PL has been examined. Intrinsic tenase purified components include 25 nM FIXa, 1 nM FVIII and 5 mM Ca++. The substrate, FX, is used at 1 μM. Any inhibitory effect of the $hsPLA_2$ has been observed on the ability of these components to activate FX under these conditions, even at $hsPLA_2$ concentrations up to 10 μM (data not shown). Thus, the PL-independent anticoagulant action of hsPLA2 is specific for the prothrombinase complex.

An inhibition of the prothrombinase generation has been observed in the absence of FVa but in the presence of PL. This may result from an inhibition of the binding of FXa to PL vesicles due to an interaction of hsPLA2 with FXa, or PL vesicles, or both (data not shown).

To investigate whether the observed inhibition of prothrombinase complex formation by $hsPLA_2$ is caused by an effect on the activation of FV by α-thrombin or by FXa, several studies of FV-activation are performed. First, 300 nM FV is preincubated for 30 min with or without 3.5 μM hsPLA2, and then activation is started by the addition of 1 nM thrombin. Second, 3 nM FV is incubated for 30 min with or without 3.5 μM hsPLA2 in the presence of 25 μM PL, and activation is started by the addition of 6 nM FXa. In both cases, the formation of FVa is determined as a function of time in a prothrombin time assay using FV deficient plasma. It has been observed that $hsPLA_2$ is unable to inhibit FV-activation by either thrombin or FXa/PL (data not shown). This suggests that $hsPLA_2$ may specifically inhibit prothrombin activation by inhibiting the formation of the prothrombinase complex.

Taken together, these results indicate that $hsPLA_2$ grII, by affecting FXa, is a potent inhibitor of prothrombinase activity, under conditions where the prothrombinase complex activity is suboptimal.

EXAMPLE 3

Reversal of $hsPLA_2$ grII inhibition on prothrombinase activity by the presence of FVa

Interestingly, when optimal conditions are used to measure prothrombinase activity (i.e., 20 pM FXa, 20 pM FVa, 5 μM PL and Ca++), a loss of the inhibitory action of hsPLA2 grII is observed (data not shown).

To further understand the lack of the inhibitory effect of $hsPLA_2$ grII on prothrombinase activity in the presence of Fva and PL, the prothrombinase complex is reconstituted with FV instead of FVa (20 pM FXa, 120 pM FV, 5 μM PL, Ca++), and the effect of 3.5 μM hsPLA2 grII, preincubated with FV and Fxa, is followed during 6 minutes.

At this time, various amounts of FVa (4 pM, 20 pM or 200 pM) are added to the prothrombinase complex. Consistent with previous experiments (Fig. 3A and 3A bis), no addition of FVa allowed a strong inhibition of prothrombinase activity by hsPLA2 grII (Fig. 6 and 6 bis, -▲-). Addition of increasing amounts of FVa amounts after 6 minutes, lead to a dose-dependent counteraction or reversal of the $hsPLA_2$ grII anticoagulant effect (Fig. 6 and 6 bis).

It should be noted in figures 6 and 6 bis that in the presence of $hsPLA_2$ grII and FV but in the absence of additional FVa, the maximal rate of thrombin generation indicated by the slope of the prothrombinase activity curve, eventually reaches that of the prothrombinase complex obtained in the absence of $hsPLA_2$ grII after a prolonged lagtime. The prothrombin activation curve in the presence of $hsPLA_2$ grII is shifted to the right along the x-axis, merely caused by an increased lagtime of prothrombinase complex activity, leading to the suggestion that the assembly of the prothrombinase complex is delaying but eventually is formed and yielded the same final activity. Using the same rationale as described above, this observation can be easily explained: whereas $hsPLA_2$ grII is effectively able to inhibit prothrombinase activity in the presence of FV, traces of prothrombin activation will provide enough thrombin to activate FV, yielding high enough levels of FVa to subsequently counteract the inhibitory effect of $hsPLA_2$ grII. To rule out the possibility that the lag is due to proteolytic destruction of $hsPLA_2$, SDS-PAGE has been used to show that the $hsPLA_2$ is not cleaved by either FXa or thrombin during the prothrombinase complex activity measurements (data not shown).

Finally, it has been tested if $hsPLA_2$ grII is able to inhibit prothrombinase activity in the presence of FXa, FVa, Ca++, and in the absence of PL. A 50%-inhibition is observed at 3.5 μM $hsPLA_2$ grII (data not shown) comparable to the effect of $hsPLA_2$ grII in prothrombinase assays

performed in the presence of FXa, FV, Ca++, and in the absence of PL.

The $hsPLA_2$ grII is thus able to inhibit the prothrombinase activity in all suboptimal conditions tested even if FVa is present. However, when prothrombinase complex reaches optimal conditions (FXa, FVa, PL, Ca++) the presence of FVa reverses the inhibitory effect of $hsPLA_2$ grII. These results suggest that hsPLA2 grII either may bind to FXa at the same site(s) as FVa, or that the binding of FVa to FXa may modify the structure of FXa leading to the dissociation and the removal of $hsPLA_2$ grII.

EXAMPLE 4

Binding of $hsPLA_2$ on FXa

The direct association of $hsPLA_2$ and FXa is demonstrated using isothermal titration calorimetry (ITC). Figure 8 (top panel) shows original data from the calorimetric titration of a 1.4 ml solution containing 4.1 μM of FXa with a solution containing 45.7 μM of $hsPLA_2$ at 37°C. Two kinetic phases are associated with each injection. Immediately following injection, an initial exothermic phase (negative numbers), which will refer to as site binding, is observed. This is followed by a smaller and slower endothermic phase (positive numbers) suggesting that a slow conformational rearrangement takes place after binding. As the number of injections increases, binding sites become saturated since the final exothermic peaks decrease in size and remain nearly constant with further injections of $hsPLA_2$. The corresponding binding isotherm in Fig. 8 (filled squares, bottom panel) shows the association transition. A least-squares estimate of association parameters using a single-site binding model gave N = 0.95 + 0.10, $\Delta H°$ = - 4.56 + 0.08 kcal/mole, $\Delta S°$ = 15.8 + 0.8 cal/mole/°K ($\Delta H°/\Delta G37°$ = 48%), and K_d = 230 ± 30 nM. These results (Fig. 8) are confirmed by titration experiments performed at lower concentrations of FXa (1.5 μM and 3.5 μM; data not shown). Therefore, these experiments show that $hsPLA_2$ and FXa bind with a small change in enthalpy ($\Delta H°$) in a 1:1 stoichiometry. The K_d

value is in between the K_d values reported for the FVa-FXa complex in the absence of PL (0.8 μM) and in the presence of PL (1 nM) (Pryzdial et al., 1991 ; Krishnawamy et al., 1990).

EXAMPLE 5

Effect of Ca++ on kon and koff rate constants of FXa/hsPLA$_2$ interaction

The inhibitory effect of hsPLA$_2$ on FXa activity, measured in FXa-one-stage assays, is mainly observed in the presence of Ca++ (Fig.1 and 2). Based on this observation, the influence of Ca++ on FXa/hsPLA$_2$ binding affinity is tested using surface plasmon resonance (SPR), studies which require less protein material than ITC. SPR allows measurements of the association rate constant (k_{on}) and of the dissociation rate constant (k_{off}) of hsPLA$_2$ to immobilized FXa. The ratio of the rate constants measured by SPR provides an apparent dissociation constant ($K_d^{app} = k_{off}/k_{on}$) that is an estimation of the equilibrium dissociation constant in solution (K_d) measured by ITC. The K_d^{app} value is usually smaller than the equilibrium K_d value as SPR rate constants measurements are performed far from equilibrium, and with one immobilized protein which decreases the overall entropy of the association reaction with respect to free protein association in solution.

Effects of Ca++ on k_{on} and k_{off} for FXa/hsPLA$_2$ association are given below in Table 1.

Table 1 : Determination of k_{on} and k_{off} rate constants for $hsPLA_2$ interaction with immobilized FXa using surface plasmon resonance.

	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	$K_d^{app} = k_{off}/k_{on}$ (nM)
$hsPLA_2$	190,000 ± 40,000	0.017 ± 0.001	86
$hsPLA_2$, Ca^{++}	530,000 ± 120,000	0.0022 ± 0.0003	4.2

Legend of Table 1 :Human FXa is covalently coupled on a sensor chip and rate constants of the $hsPLA_2$/FXa interaction are determined as described in "Methods". Values are the mean ± sem of three independent experiments.

Table 1 shows that $hsPLA_2$ binds to FXa immobilized onto the sensor chip surface in the absence of Ca++. However, 5 mM Ca++ in the screening buffer decreases koff nearly ten fold and increases kon nearly 3 fold, leading to a 30 fold increase in K_d^{app}. Therefore, data in Table 3 (see example 6) demonstrate that Ca++ allows the formation a higher affinity FXa/$hsPLA_2$ complex.

EXAMPLE 6

Inhibition of prothrombinase activity by peptides related to the region 51-74 of the $hsPLA_2$ grII

The effect of a synthetic peptide 51-74 corresponding to a basic region of the $hsPLA_2$ grII (see Table 2 for the amino acid sequence of the whole molecule) has been investigated as well as three 12-mer derived peptides (see Table 3). The scrambled peptide 51-74 is used as control.

Table 2 : Amino acids sequences of hsPLA$_2$ grII and two synthetic peptides.

hsPLA$_2$ grII :

1 10 20 30 40 50

NLVNFHRMIKLTTGKEAALSYGFYGCHCGVGGRGSPKDATDRCCVTHDCC

60 70 80 90 100

YKRLEKRGCGTKFLSYKFSNSGSRITCAKQDSCRSQLCECDKAAATCFAR

110 120

NKTTYNKKYQYYSNKHCRGSTPRC

*

peptide 51-74 : Ac-YKRLEKRGSGTKFLSYKFSNSGSR-NH$_2$
(net charge +6)

scrambled *
peptide 51-74: Ac-GFYSKGSLSRTRFYKGNKESKLRS-NH$_2$
(net charge +6)

Legende of table 2 : In the sequence of hsPLA$_2$ (Kramer et al., 1989), the amino acids of the active site are indicated in bold. The synthetic peptide 51-74 represents the basic region of the hsPLA$_2$ (residues 51-74) with the cysteine 59 replaced by a serine (indicated by an asterisk), to avoid disulfide formation between peptide molecules. For the two peptides, basic residues are shown in bold and acidic residues are underligned.

Table 3: Inhibition of prothrombinase activity by selected peptides with sequences of the basic region of residues 51-74 of hsPLA$_2$ grII

Peptide name	Sequences	NaCl (M)	IC_{50} (μM)
peptide 51-74	YKRLEKRGSGTKFLSYKFSNSGSR	0.10	8 ± 1
		0.22	8 ± 2
scrambled peptide 51-74	GFYSKGSLSRTRFYKGNKESKLRS	0.10	70 ± 5
		0.22	>200
peptide 51-62	YKRLEKRGSGTK	0.10	18 ± 2
		0.10	22 ± 3
		0.22	20 ± 3
		0.22	22 ± 3
peptide 59-70	SGTKFLSYKFSN	0.10	>200
		0.22	ND
peptide 63-74	FLSYKFSNSGSR	0.10	>200
		0.22	ND
peptide reverse 62-51	KTGSGRKELRKY	0.10	>200
		0.22	ND
peptide D-51-62 (all D)	YKRLEKRGSGTK	0.10	>200
		0.22	ND

Legend of Table 3 : Prothrombinase activity assays are performed as described in "Methods". Reactants (20 pM FXa, 120 pM FV, 5 μM PL and 5 mM Ca++) are preincubated in the presence of various concentrations of the indicated peptides. After 8 min preincubations, the prothrombinase

activity is determined and expressed in percent of the prothrombinase activity measured in the absence of peptide. Then, the percent of residual prothrombinase activity is analyzed as a function of the peptide concentration, and the IC50 value is calculated. ND : not determined

When preincubated with FXa and FV, we observe a dose-dependent inhibition of prothrombinase activity (FXa, FV, PL, Ca++) by peptide 51-74, with an IC50 value of 8 ± 1 μM (table 3). In order to better define the part of the molecule involved in the inhibitory action, the effect of smaller overlapping peptides derived from this basic region of the $hsPLA_2$ grII has been investigated. Only the peptide 51-62 inhibits prothrombinase activity, as showed in the table 3, with an IC50 value (18 ± 2 μM or 22 ± 3 μM) close to that obtained with larger peptide 51-74. The scrambled peptide shows a nonspecific inhibitory effect when testing with 0.1 M NaCl (IC50 of 70 ± 5 μM) since it is lost when higher salt concentration (0.22 M NaCl) is used (Table 2). This supports the idea that the inhibitory effect of scrambled peptide at 0.1 M NaCl concentration is due to its basic nature (net charge +6) but does not imply specific residues. In contrast, the inhibition of prothrombinase activity by peptides 51-74 and 51-62 is specific since it is maintained with similar efficiency under 0.22 M NaCl concentration (Table 3). A 12-mer reversed peptide 62-51 is devoid of inhibitory effect, as is the control peptide D-51-62 with all amino acid residues in a D-configuration. Thus the stereospecific L-conformation of amino acids in peptide 51-62 is required for the inhibition of FXa/FVa complex formation.

These results indicate that the basic region (residues 51-74) of hsPLA2, and particularly residues 51-62, specifically inhibits prothrombinase activity, and is most likely responsible for the inhibition of FXa/FVa complex formation. These basic region may therefore represent the part of the molecule being involved in the inhibition of FXa. It is important to note that residues 51-74 of the molecule are not implicated in the catalytic

activity and that the same region has been postulated to be involved in the anticoagulant effect of sPLA2 from snake venom.

It has been recently observed that $hsPLA_2$ grII, secreted during platelet activation, exhibits anticoagulant activity. In this way, it might exert a negative feedback regulation on coagulation, which would avoid an excessive procoagulant effect of activated platelets (Mounier et al., 1996). It has been demonstrated that this inhibitory effect does not require the enzymatic activity of the enzyme, indicating that hsPLA2 grII may interact and have pharmacological effects on non-phospholipid targets. This is in good agreement with results of Ouyang et al. (1978), Stefansson et al. (1990) and, more recently, Babu and Gowda (1994), who have already suggested that some venom sPLA2 are able to affect blood coagulation by mechanisms that do not involve their catalytic activity. However, the targets of the venom enzymes were not identified.

Prothrombine time coagulation assays indicate that $hsPLA_2$ grII does not inhibit FVa activity, while FXa-one-stage coagulation assays show an inhibition of FXa activity, particularly in the présence of Ca++. Thus, $hsPLA_2$ grII is able to inhibit FXa activity in clotting assays. The inhibitory action of $hsPLA_2$ grII on prothrombinase activity was also observed by Inada et al. (1994), although the molecular mechanism was still not clear. The results indicate that $hsPLA_2$ grII is able to inhibit prothrombinase activity, especially when the conditions are not optimal for prothrombinase complex activity or assembly. It has been demonstrated that in the absence of FVa or PL, dependent on the preincubation of hsPLA2 grII with FXa, $hsPLA_2$ grII effectively downregulates prothrombinase activity (Fig. 3 and 4) and that in the absence of PL and in the presence of FVa, preincubation of $hsPLA_2$ with FXa effectively downregulates prothrombinase generation (Fig. 5). However, if FV is present instead of FVa, $hsPLA_2$ grII inhibites prothrombinase activity only until there is enough in situ

FVa generated by traces of thrombin/FXa to optimize prothrombinase activity conditions and overcome $hsPLA_2$ (Fig. 6 and 6 bis). The activity of the intrinsic tenase complex (FIXa, FVIII, Ca++) is unaffected by $hsPLA_2$, although this coagulation complex shares common characteristics with the prothrombinase complex (associated with the structural homology of FVa and FVIIIa, and of FIXa and FXa). Moreover, activation of FV by either FXa or thrombin is not inhibited by $hsPLA_2$. These observations indicate that the inhibitory action of $hsPLA_2$ is likely to be specific for the prothrombinase complex or for its formation.

Mounier at al. (1996) report indicated that 74 nM $hsPLA_2$ grII was unable to inhibit prothrombinase activity in the absence of FV, leading to the suggestion that FV might be the target of the $hsPLA_2$ grII. It has now observed that $hsPLA_2$ grII still remains a potent inhibitor of prothrombinase activity in the absence of FV but for higher concentrations (IC50 of 3.5 μM) that in the presence of both FV and PL. This discrepancy is easily explained by the higher amounts of FXa needed to measure prothrombinase activity in the absence of FV (20 nM compared to 20 pM).

These results indicate that $hsPLA_2$ grII may bind to FXa at the same site(s) as FVa, but with a lower affinity and thereby may decrease the prothrombinase activity by inhibiting the formation of a FXa/FVa complex. This is supported by the demonstration that $hsPLA_2$ binds to FXa with a 1:1 stoichiometry and a K_d value of 230 nM (Fig.8). Although not necessary for the interaction, the Ca++ increases the k_{on} rate constant and decreases the k_{off} rate constant, leading to a higher affinity of $hsPLA_2$ for FXa (Table 1). The prothrombinase complex has a catalytic efficiency in the activation of prothrombin that is several orders of magnitude higher than FXa acting alone. In the absence of PL, the FVa-FXa interaction is governed by a K_d of 0.8 μM and is dependent on the presence of Ca++ (Pryzdial and Mann, 1991). In the presence of PL vesicles and Ca++, the K_d of the FVa-FXa complex decreased to approximatively 1 nM (Krishnaswany et al., 1990). This may

explain why, under suboptimal conditions where FVa is added in the absence of PL, FVa is unable to remove the $hsPLA_2$ grII inhibitory effect, since in this case its affinity for FXa is lower than that of $hsPLA_2$ for FXa. Another hypothesis to explain the absence of inhibition by $hsPLA_2$ grII in the presence of FVa is that on binding, FVa induces a conformational change of FXa which then loses its ability to bind $hsPLA_2$ grII. It has been previously reported that $hsPLA_2$ possesses an anticoagulant activity in whole plasma, as demonstrated by the increase of its recalcification time (Cirino et al., 1993), and that this anticoagulant activity in plasma is even observed when activated platelets are present (Mounier el al., 1996). hsPLA2 is thus able to produce an inhibitory effect on blood coagulation under experimental conditions occurring during clot formation. Moreover, it is well established that the level of hsPLA2 in serum is strongly increased, from 0.35 nM up to 0.6 μM, under various pathological states associated with inflammation, as in the case of acute pancreatitis, multiple organ failure, septic shock or rheumatoid arthritis (Nevalainen et al., 1993 ; Nyman et al., 1996 ; Rintala et al., 1995 ; Komatstubara et al., 1995). Blood platelets secrete large amount of hsPLA2 upon activation (Kramer et al., 1989 ; Mounier et al., 1995), and it is likely that high local concentrations of this enzyme are reached during stasis platelet activation and secretion or during local thrombolytic events, even if $hsPLA_2$ levels are more difficult to assess than in serum (Fourcade et al., 1995). These results show that the $hsPLA_2$/FXa interaction is governed by a K_d of 230 nM, a value fully consistent with a potential localized and/or systemic anticoagulant role of $hsPLA_2$ during various physiologic or physiopathological states. In conclusion, These results strongly indicate that $hsPLA_2$ grII exhibits inhibitory action on FXa under suboptimal conditions for prothrombinase activity, an action that will be lost under extensive thrombolytic events when high amounts of FVa are produced.

These results suggest that $hsPLA_2$ grII released by activated platelets during primary hemostasis may act as a negative modulator of thrombin generation by preventing the initial prothrombinase complex assembly. It has been already mentioned that the K_d value of $hsPLA_2$/FXa interaction (230 nM) is higher than the one of FVa/FXa interaction (1 nM). For equal concentrations of $hsPLA_2$ and FVa, these K_d values would favor FXa/FVa interactions. However, at the initiation stage of coagulation, when only very low amounts of FVa are present (the precursor form of FVa, FV, being the major form circulating in plasma or stored into α-granules of platelets), the advantage would favor $hsPLA_2$/FXa interactions. In agreement, the original negative role of $hsPLA_2$ is pointed out, since its effect will be significant at early stages of the coagulation process until enough FVa is generated. The function of $hsPLA_2$ will be therefore to inhibit coagulation by delaying the formation of a fully active prothrombinase complex, rather than to inhibit the activity of the fully active prothrombinase complex on prothrombin.

Beside a common enzymatic mechanism (Ami and Ward, 1996), it has been reported that $sPLA_2$ exert different pharmacological effects (Kini and Evans. 1989). The presence of distinct regions on the molecule, described as "pharmacological sites" and not implicated in the catalytic activity, may explain the diversity of these pharmacological effects (Kini and Evans, 1989). In particular, Kini and Evans (1987) have proposed that the anticoagulant effect of some venom sPLA2 was correlated to a basic region located between the residues 50-80. The $hsPLA_2$ grII have seven basic amino acids (K or R) located between residues 51-74, and three-dimensional studies have shown that this part of the $hsPLA_2$ grII is exposed at the surface of the molecule and is not associated with the catalytic site (White at al., 1991 ; Wery et al., 1991). Thus, this region appears to be a good candidate to be involved in the inhibition of FXa by $hsPLA_2$ grII. To test this hypothesis, the inhibitory effect of a synthetic

peptide 51-74 has been examined on prothrombinase generation. The peptide 51-74 shows an inhibitory effect with an IC50 of 8 μM. This inhibition is specific, since the scrambled peptide 51-74 is less potent at 0.1 M NaCl and completely devoid of detectable activity at 0.22 M NaCl. The moderable inhibition of prothrombinase activity by scrambled peptide 51-74, observed at low ionic strength is likely nonspecific due to its basic nature (net charge +6).

Furthermore, the smaller peptide 51-62 is also able to inhibit prothrombinase, suggesting that the region of residues 51-62 of $hsPLA_2$ is involved in the inhibition of prothrombinase generation by interacting with FXa. Reversed peptide 62-51 and peptide (D)51-62 are unable to inhibit prothrombinase activity up to 200 μM, supporting the specificity of the interaction of peptide 51-62 with FXa. Thus, the region 51-74, and more precisely the region 51-62, of the hsPLA2 grII is independently able to inhibit prothrombinase activity duo to a specific mechanism. The importance of the 51-62 region in the binding of $hsPLA_2$ on FXa points out the presence of basic clustered residues which might be critical for the interaction. In agreement, it has been shown that substitution of Lys^{56} by Gln in $hsPLA_2$ reduced the antiprothrombinase activity of the enzyme, whereas substituting Asp^{59} by Arg in porcine pancreatic $sPLA_2$ increased this activity (Inada et al., 1994). The thermodynamic characterization of the $hsPLA_2$-FXa association reaction reported here also agrees with this conclusion. $hsPLA_2$ binding to FXa in solution presents a favorable but small binding enthalpy ($\Delta H°$ = - 4.56 kcal/mole) and a favorable and significant entropic contribution to binding ($T\Delta S$ = + 4.90 kcal/mole). The small enthalpic contribution may account for the short amino acid sequence size at the binding site, while the favorable entropic contribution to binding may reflect binding induced desolvation and/or anion release upon binding at the level of the basic sequence of residues 51-62 of

hsPLA$_2$. Heeb et al. (1996) suggested that one binding site for FXa involves residues 493-506 in FVa (GLLLICKSRSLDRR), which shows some similarity (bold letters) to the peptide 51-62 (YKRLEKRGSGTK) when basic residues are compared. Finally, this part of the hsPLA$_2$ grII molecule (CYKRLEKRGSGTK) also shares similarities with the equivalent region on FVIII (LLICYKESVDQRG). It is thus tempting to speculate that hsPLA$_2$ grII would be able to inhibit the tenase activity by similar mechanism with FVIII and FIXa.

This remarkable homology supports the hypothesis that hsPLA$_2$ acts by competing with FVa for binding to FXa. It is not at all clear which parts of the FXa molecule is targetted by FVa, and thus it is also very difficult to establish which FXa regions interact with hsPLA$_2$. The most probable hypothesis is that hsPLA$_2$ binds to the same site as FVa on FXa, but a noncompetitive mechanism can not be exclude due to the binding of clustered basic residues of hsPLA$_2$ to specific negatively charged residues present in γ-carboxyglutamate-rich domain of FXa. However, whatever the part of FXa that interacts with hsPLA$_2$, this interaction does not affect the active site of FXa, since the enzymatic activity of FXa alone is not affected. Site-directed mutagenesis studies, as well as chemically synthesized hsPLA$_2$ variants (Hackeng et al., 1997), will help to analyze which amino acids of the region 51-62 are crucial for the anticoagulant activity of hsPLA$_2$, and a three tridimensional structure of the FXa/hsPLA$_2$ complex will allow to identify the interacting region of FXa.

In conclusion, the observation that hsPLA$_2$ binds to FXa and delays fully active prothrombinase generation apparently by preventing FVa/FXa interactions, provides a molecular mechanism explaining the nonenzymatic anticoagulant effect of hsPLA$_2$. Based on this work and on the purification and cloning of a protein receptor for sPLA$_2$ (Lambeau et al. 1994 ; Ishizaki et al., 1994), it is clear that sPLA$_2$ exerts biochemical effects on protein

targets through pharmacological sites that are distinct from the $sPLA_2$ active site.

United States Provisional Application Serial No. 60/048,668, filed in the U.S. Patent and Trademark Office on June 5, 1997, is incorporated herein by reference in its entirety.

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CLAIMS

1. A peptide comprising at least eleven amino acids numbered 51 to 62 of hsPLA$_2$ gr II sequence.

2. A peptide according to claim 1 comprising amino acids numbered 51 to 74 of hsPLA$_2$ gr II sequence.

3. A peptide exhibiting an anticoagulant effect corresponding to an amino acid chain containing at least a seven consecutive amino acid from the numbered 51 to 62 amino acid sequence of hsPLA$_2$ gr II.

4. A peptide exhibiting an anticoagulant effect corresponding to an amino acid chain containing at least 14 amino acids having at least 50% amino acids identity with the numbered 51 to 74 amino acid sequence of hsPLA2 gr II.

5. A peptide according to any one of the claims 1 to 4, wherein said peptide is able to inhibit prothrombinase activity.

6. A peptide according to claim 5, wherein the molecular target for the anticoagulant action of said peptide is Factor Xa (FXa).

7. A peptide according to any one of the claims 1 to 6, wherein the presence of Factor Va (FVa) is capable of reversing said peptide activity.

8. A peptide according to claim 7, wherein the presence of Factor Va (FVa) is capable of reversing the activity of said peptide under optimal conditions.

9. A peptide according to claim 7 or 8, wherein said peptide is a Fva competitor.

10. Nucleotide sequence coding for a peptide according to any one of the claims 1 to 9.

11. A monoclonal or polyclonal antibody, or fragments thereof, characterized in that it binds a peptide according to any one of the claims 1 to 9.

12. A monoclonal or polyclonal antibody, or fragments thereof according to claim 11, characterized in that it inhibits hsPLA_2 gr II anticoagulant effect.

13. A pharmaceutical composition comprising a peptide according to any one of claims 1 to 9 or an antibody according to claim 11 or 12, in combination with a pharmaceutically acceptable vehicle.

14. Use of a peptide according to any one of the claims 1 to 9 or an antibody according to claim 11 or 12, in a manufacture of a medicament for the prevention or the treatment of hemostatic disorders.

15. A method of regulating the coagulant effect *in vivo* in human or in animal comprising the step of administering an effective amount of an active peptide according to any one of the claims 1 to 9, of an antibody according to claim 11 or 12, or of a pharmaceutical composition according to claim 13.

16. A method of treating or preventing thrombus formation and limiting platelet activation in vivo in human or in animal comprising the step of administering an effective amount of an active peptide according to any one of claims 1 to 9 or of a pharmaceutical composition according to claim 13.

17. A method of screening new compounds for use as a medicament for the prevention or the treatment of coagulation disorders, comprising the use of a peptide according to any one of claims 1 to 9.

18. A method of screening according to claim 17, comprising the steps of :

a) contacting a sample containing said test compound with a peptide according to any one of claims 1 to 9 ;

b) detecting the binding of said test compound with said peptide ; and

c) selecting said test compound which is able to bind with said peptide.

19. A method of screening according to claim 17, comprising the steps of :

a) contacting a sample containing said test compound with a peptide according to any one of claims 1 to 9 in conditions permitting the measure of said peptide anticoagulant effect;

b) measuring the said peptide anticoagulant effect ; and

c) selecting said test compound which is able to modify said peptide anticoagulant effect.

20. A kit for the determination of a hemostatic disorder, comprising a peptide according to any one of claims 1 to 9.

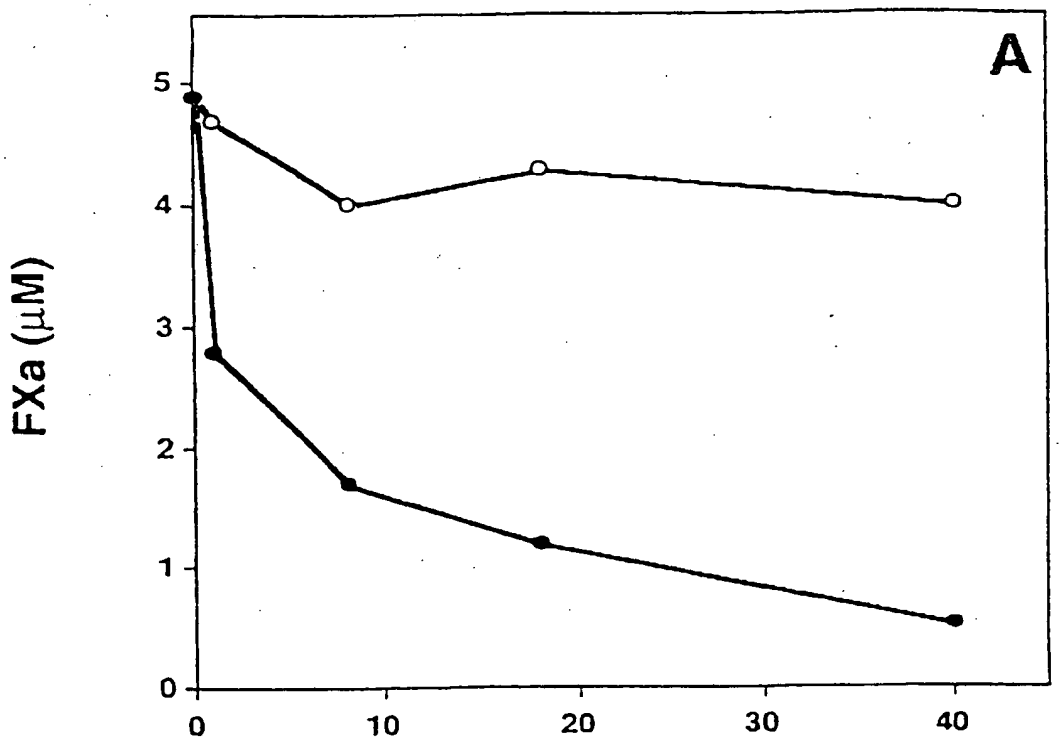

FIGURE 1A

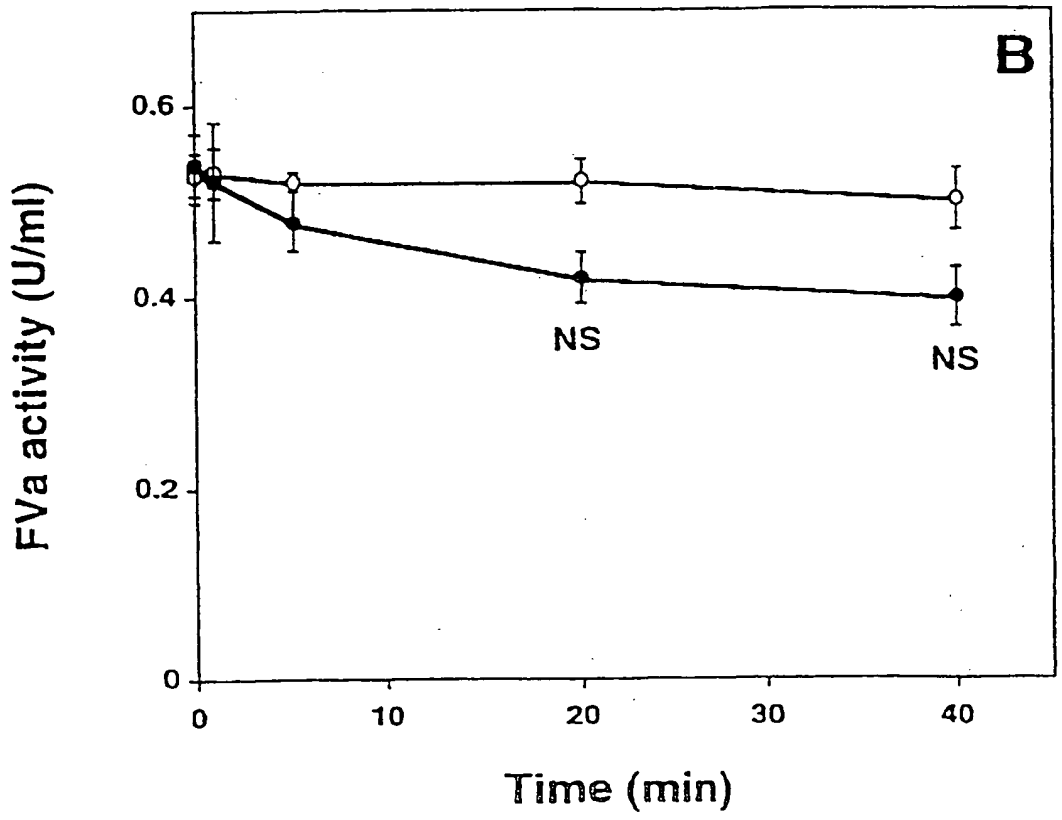

FIGURE 1B

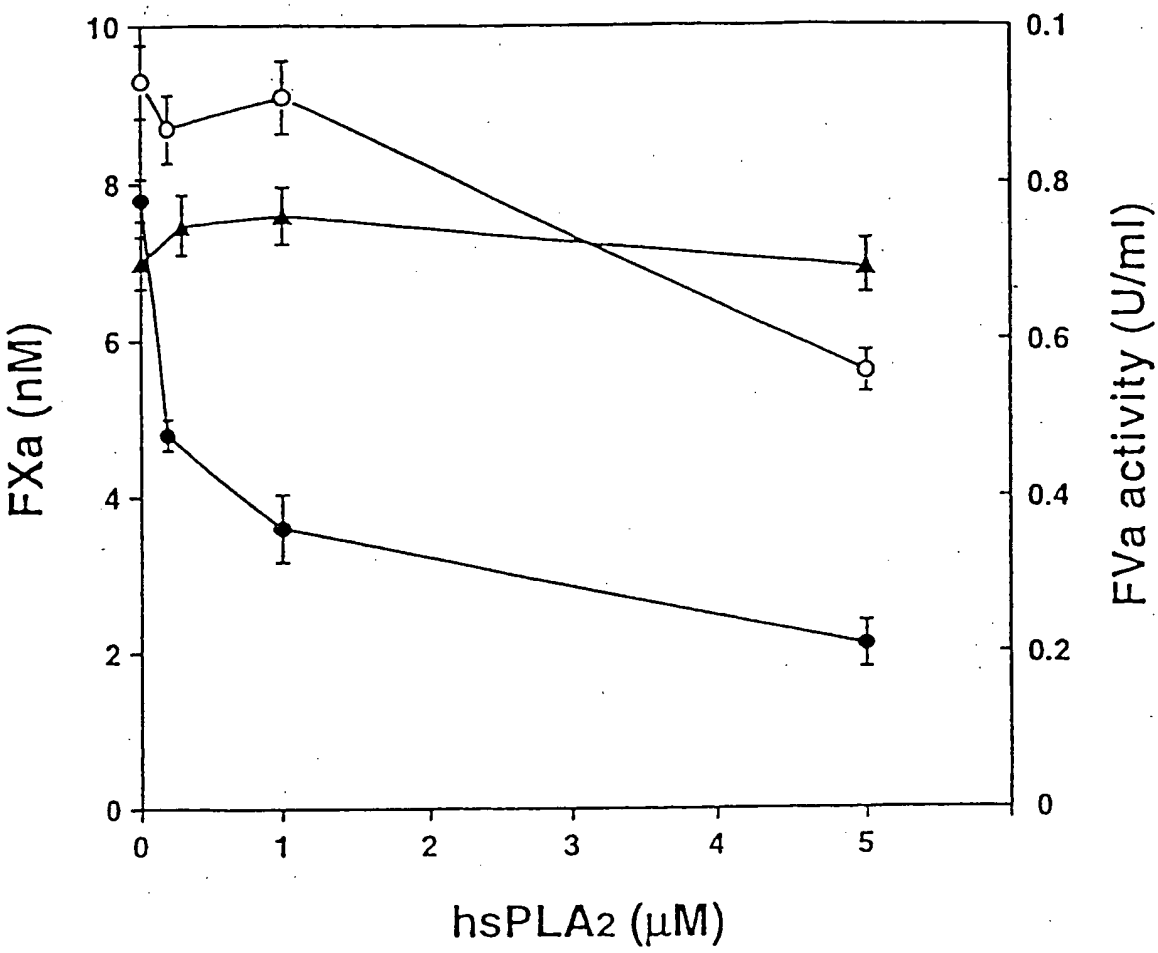
FXa (nM)
FVa activity (U/ml)
hsPLA2 (μM)
0
2
4
6
8
10
0
0.2
0.4
0.6
0.8
0.1
0
1
2
3
4
5

FIGURE 2

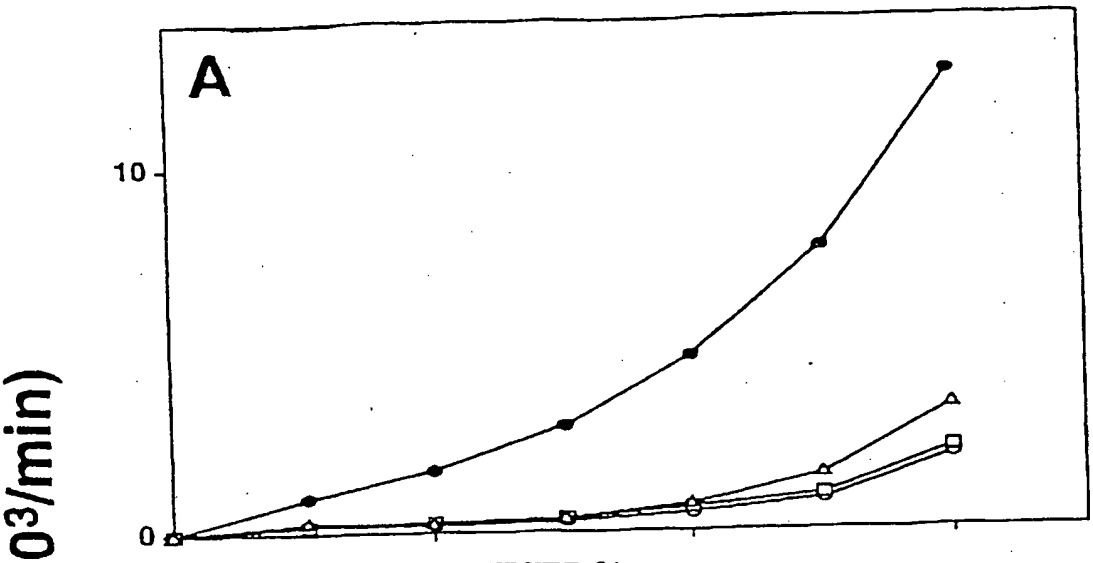

FIGURE 3A

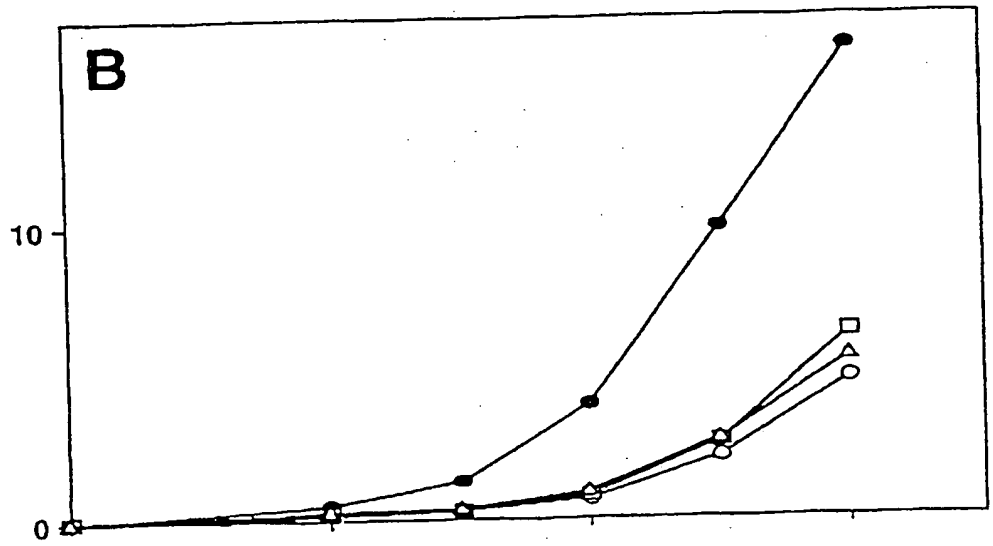

FIGURE 3B

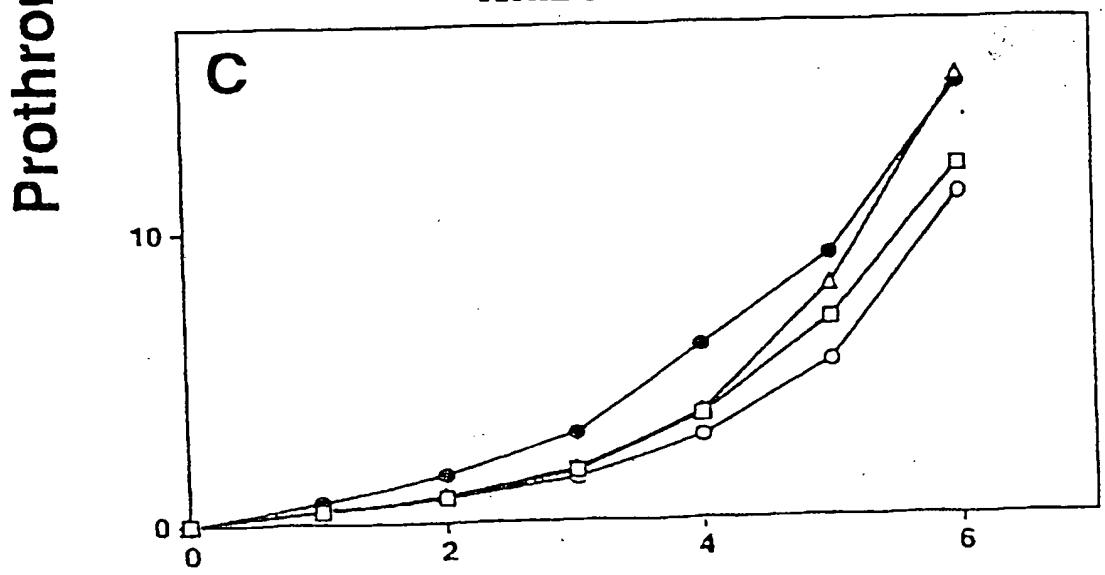

FIGURE 3C

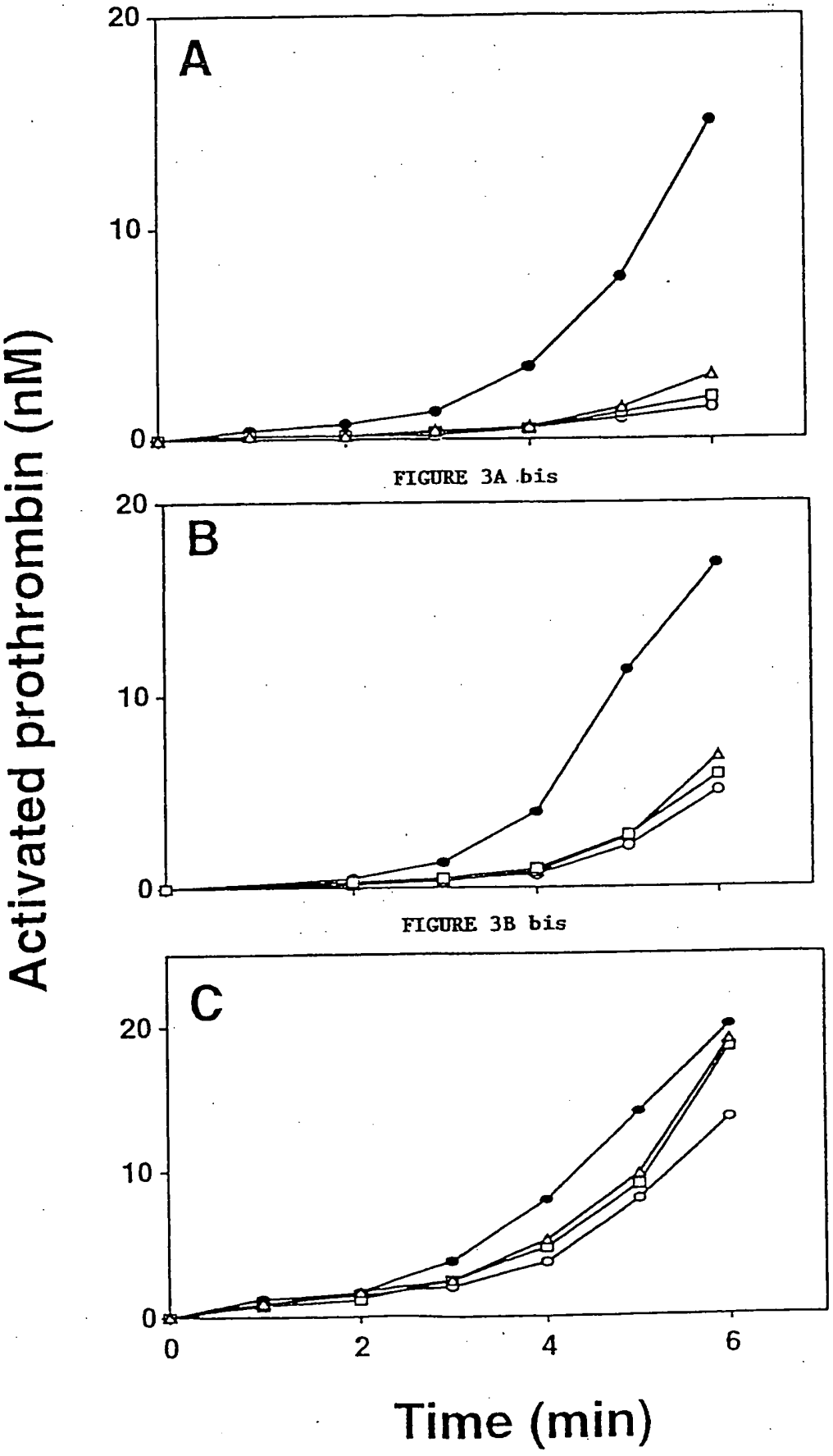

FIGURE 3A bis

FIGURE 3B bis

FIGURE 3C bis

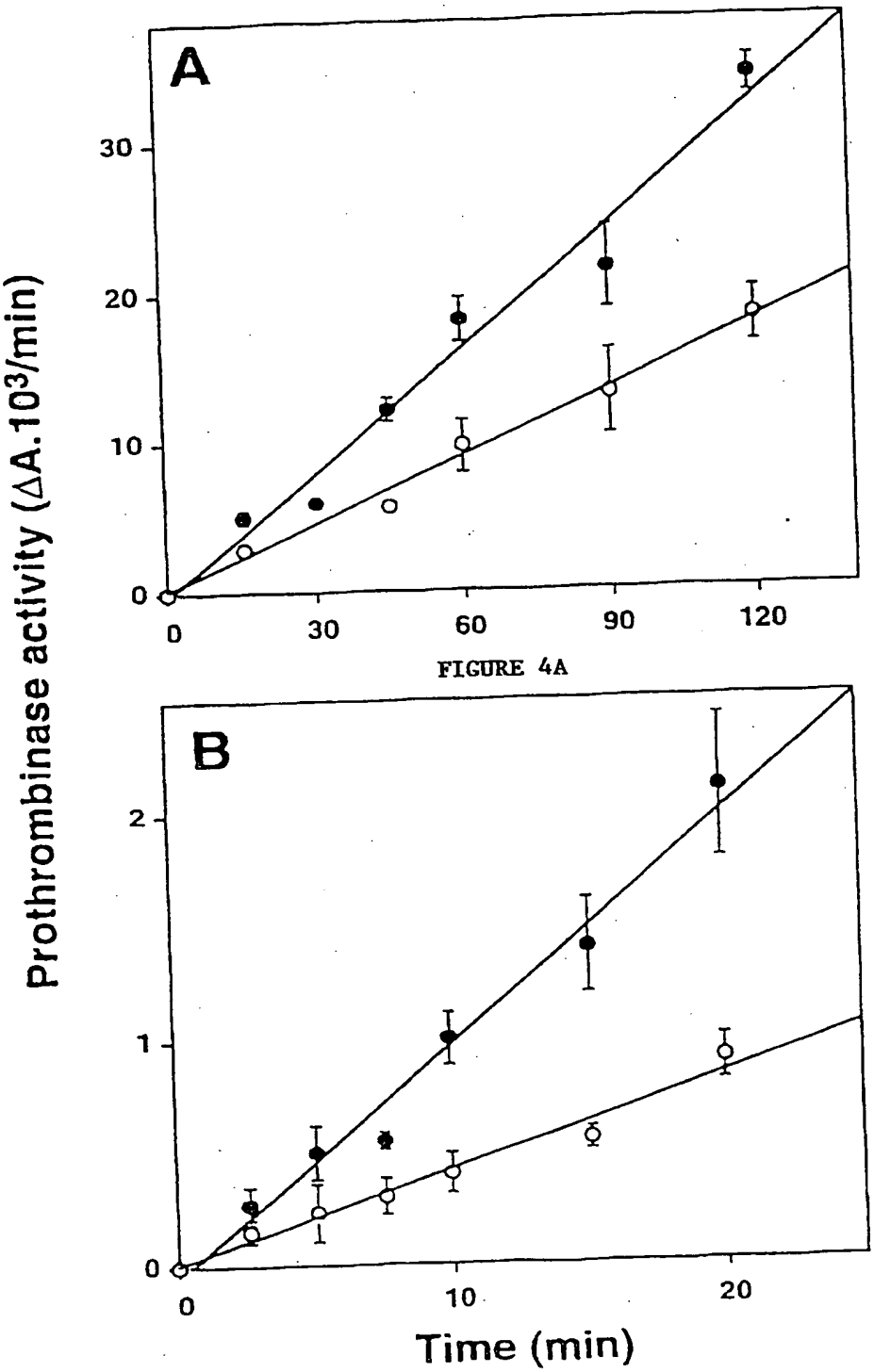

FIGURE 4A

FIGURE 4B

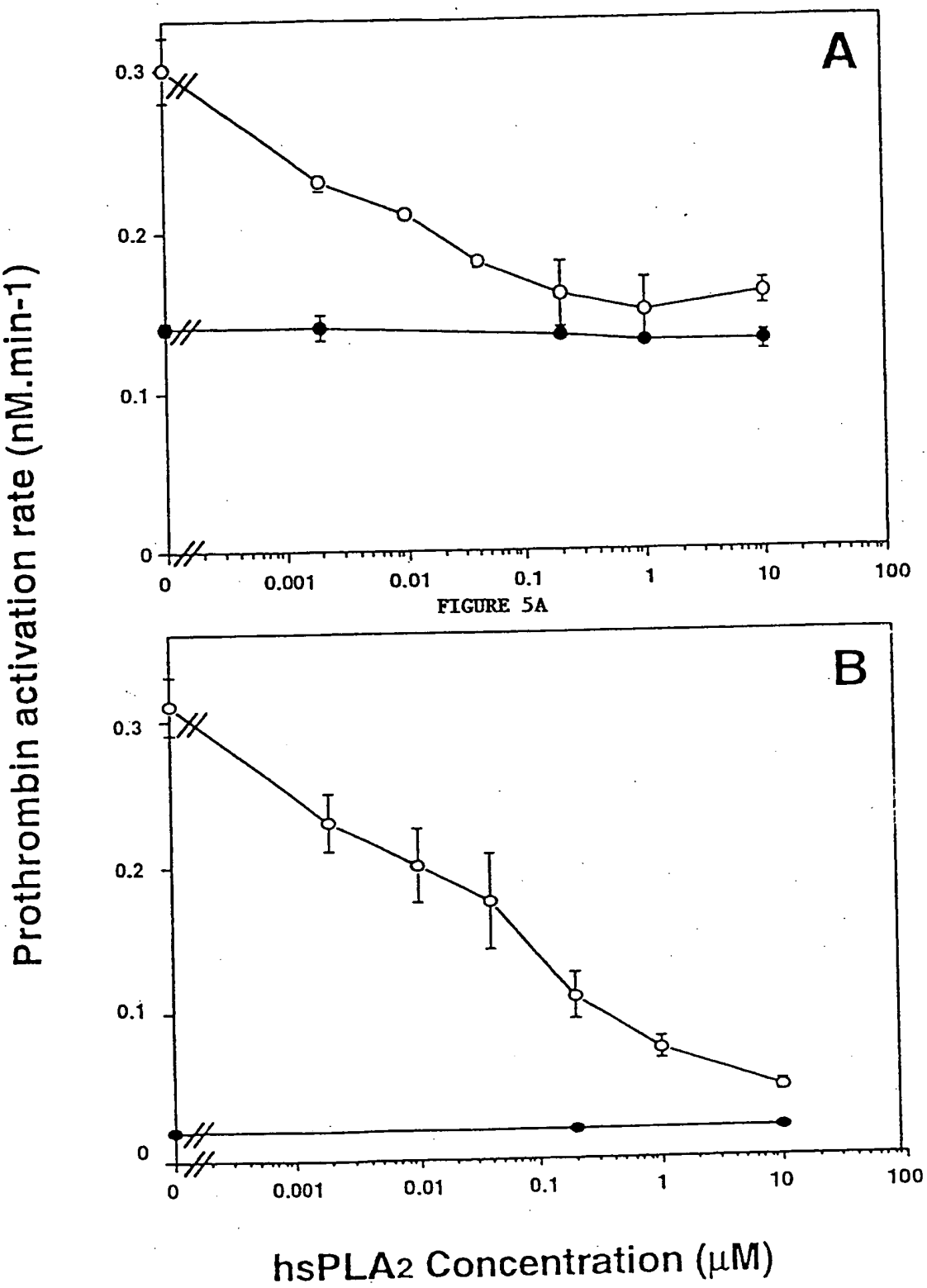

FIGURE 5A

FIGURE 5B

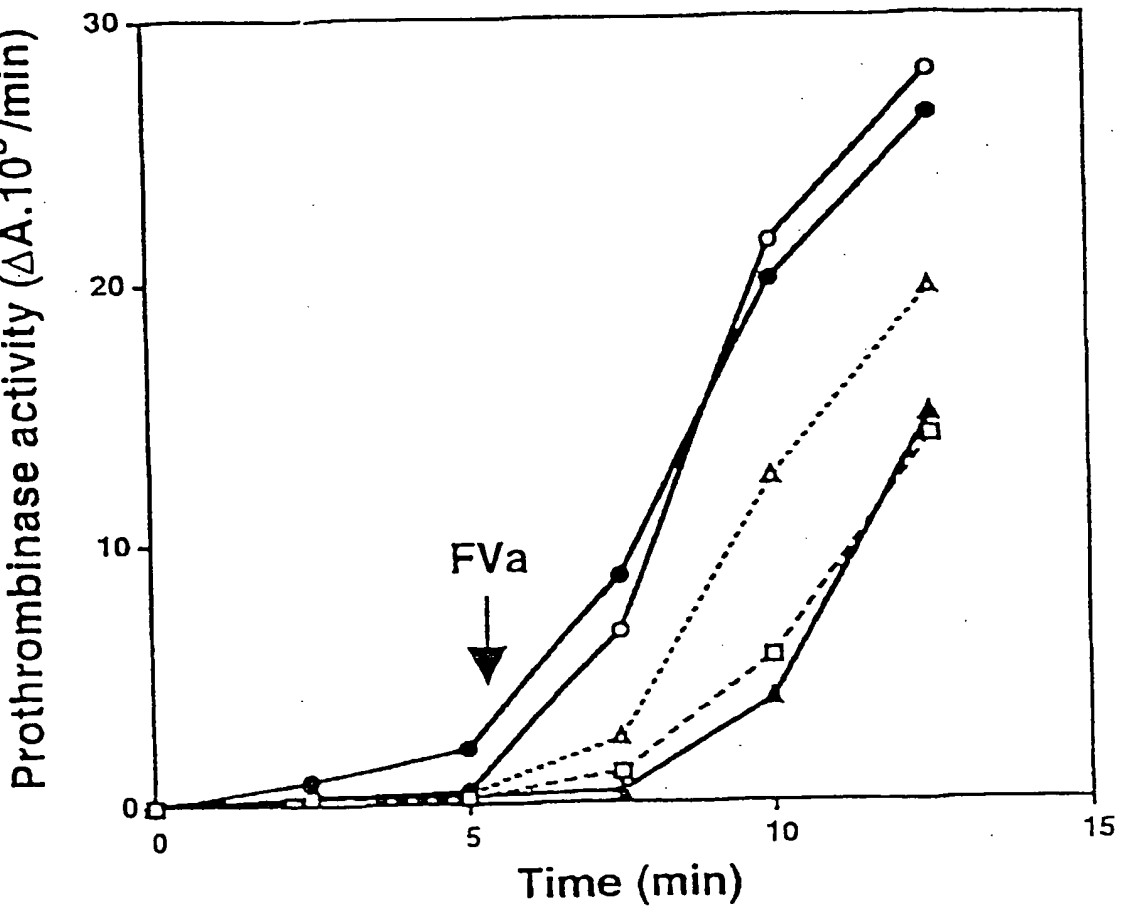
Prothrombinase activity ($\Delta A.10^3$/min)
30
20
10
0
FVa
0
5
10
15
Time (min)

FIGURE 6

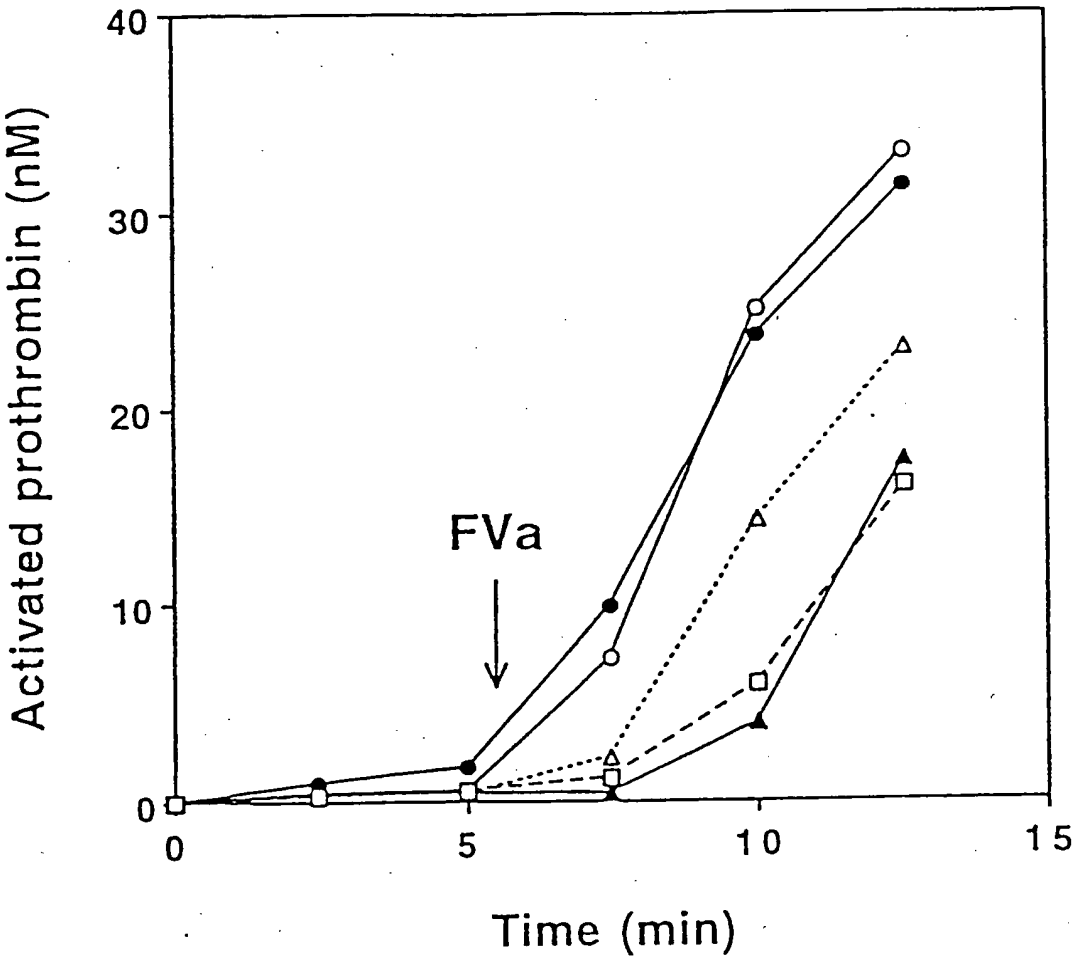

FIGURE 6bis

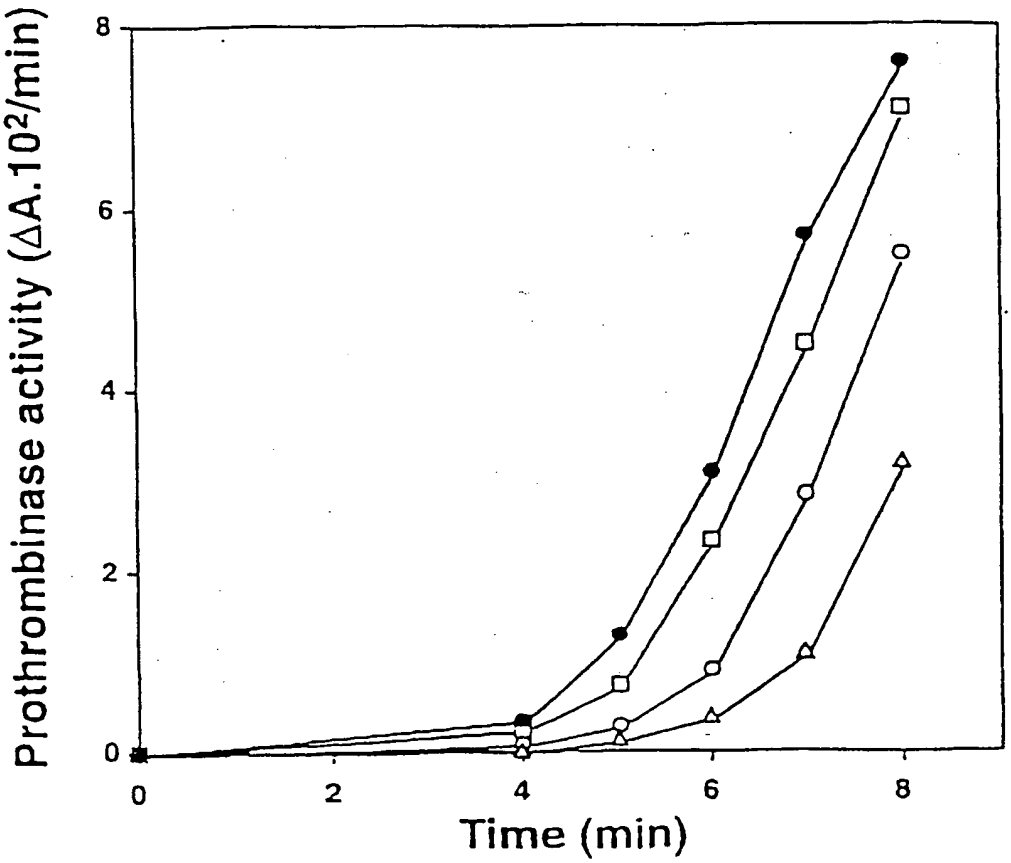

FIGURE 7

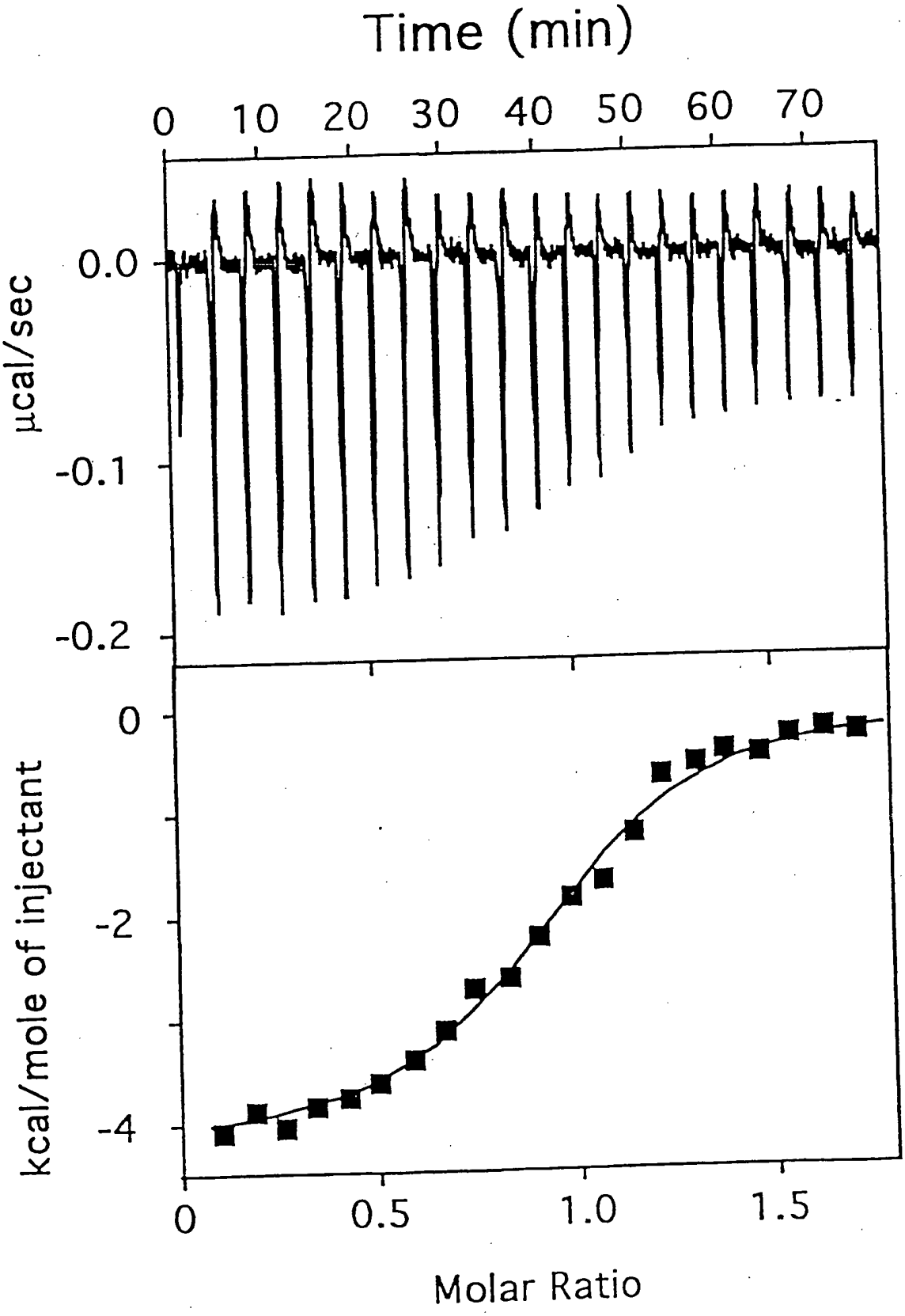
Time (min)
0
10
20
30
40
50
60
70
0.0
-0.1
-0.2
µcal/sec
0
-2
-4
kcal/mole of injectant
0
0.5
1.0
1.5
Molar Ratio

FIGURE 8

INTERNATIONAL SEARCH REPORT

Internal Application No
PCT/IB 98/00869

A CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K7/06 C07K7/08 A61K38/08 A61K38/10 G01N33/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K G01N

Documentation searched other than minimumdocumentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category '	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	EP 0 687 685 A (TEIJIN LTD) 20 December 1995 see page 5; tables 1-3 ---	1-9, 13-16
X	US 5 019 508 A (JOHNSON LORIN K ET AL) 28 May 1991 see column 10 - column 14; figure 1 --- -/--	1-20

[X] Further documents are listed in the continuation of box C

[X] Patent family members are listed in annex

' Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publicationdate of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
2 November 1998	16/11/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P B 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel (+31-70) 340-2040, Tx 31 651 epo nl, Fax: (+31-70) 340-3016	Cervigni, S

2

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INTERNATIONAL SEARCH REPORT

Intern. nal Application No
PCT/IB 98/00869

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate of the relevant passages	Relevant to claim No
X	C. MOUNIER ET AL: "The anticoagulant effect of the human secretory phospholipase A2 on blood plasma and on a cell-free system is due to a phospholipid-independent mechanism of action anvolving the inhibition of factor Va" EUR. J. BIOCHEM., vol. 237, 1996, pages 778-785, XP002082787 see abstract see page 784, column 1, paragraph 2 ---	1-20
X	D.L. SCOTT ET AL.: "Structure of free and inhibited human secretory phospholipase A2 from inflammatory exudate" SCIENCE, vol. 254, November 1991, pages 1007-1010, XP002082622 see the whole document see figure 2 ---	1-9
X	WO 89 09818 A (BIOGEN INC) 19 October 1989 see page 6, paragraph 1 see page 14-15 ---	1-20
X	WO 93 01215 A (GARVAN INST MED RES) 21 January 1993 see page 3, paragraph 1; claims; figure 1 -----	1-20

2

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB 98/00869

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1 [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely

Remark: Although claims 15-16 and at least in part 17 are directed to a method of treatment or to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2 [ ] Claims Nos
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically

3 [ ] Claims Nos.
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6 4(a)

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1 [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2 [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee

3 [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos :

4 [ ] No required additional search fees were timely paid by the applicant Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. ial Application No
PCT/IB 98/00869

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		US 5656602 A	12-08-1997